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The primary objective of these studies was to determine the neurotoxic risks of combining acetylcholinesterase inhibitors (AChEIs) with *N*-methyl-D-aspartate (NMDA) receptor antagonists. There is evidence that although NMDA receptor antagonists are considered neuroprotective against excess glutamate release during acute conditions, these same agents may also induce neurodegeneration in brain areas associated with attention and motivation; This neurodegeneration is exacerbated by muscarinic cholinergic agonists. Military personnel and civilians may be exposed to a wide array of cholinesterase inhibitors by pesticide exposure, chemical weapons deployment or prophylactic treatment. Exposure to these agents may elevate brain levels of acetylcholine (ACh), leading to increased muscarinic cholinergic stimulation. Individuals may be at risk of neuronal degeneration if they are co-exposed to a NMDA receptor antagonist, for example, cough suppressants, anticonvulsants, ethanol or the Alzheimer Disease agent, memantine. We approached this problem by using patch-clamp electrophysiology, behavioral assessment and histopathology to examine the effects NMDA receptor antagonists in animals co-exposed to AChEI agents. We found that the AChEI, PB is neuroprotective in some animals co-exposed to NMDA antagonists, but is lethal in others.

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Table of Contents

INTRODUCTION The key objective of these studies was to try to determine the underlying mechanism by which NMDA receptor antagonists produce neurotoxicity, specifically in the posterior cortex and retrosplenial cortex (PC/RSC). We also wished to determine the neurotoxic risks of combining acetylcholinesterase inhibitor (AChEI) enzymes with agents that block *N*-methyl-D-aspartate (NMDA) receptors. We approached these problems in three ways: histopathological studies, behavioral assessment, and patch-clamp electrophysiological recordings.

BODY

In vivo studies:

Introduction:

Recent events have returned to the forefront concerns that future terrorist attacks could involve chemical weapons- including nerve-gas agents. Preparations against future attacks include the identification of safe and effective measures to prevent toxicity against these weapons: including the use of prophylactic agents such as pyridostigmine bromide (PB), and the use of anticonvulsants to treat exposed individuals. One class of compounds that may exacerbate the toxicity of AChEIs, are NMDA receptor antagonists. Evidence for possible neurotoxic interactions between these agents was provided by an important study by Olney and colleagues (Corso et al., 1997), who found that the muscarinic cholinergic agonist, pilocarpine, greatly exacerbated the neurotoxicity of NMDA receptor antagonists. However, it was not clear if AChEIs could also exacerbate NMDA receptor antagonist-mediated neurotoxicity by altering levels of the endogenous agonist, acetylcholine (ACh). If so, this could have wide-ranging military and civilian repercussions, given the number of different AChEI agents to which one might be exposed.

There are a broad range of drugs and investigational agents that have been found to possess NMDA receptor antagonist activity. For some drugs, this characteristic may be a major mechanism for the therapeutic agent (eg. the anesthetic agent, ketamine or the research tool, MK-801). For other drugs, NMDA receptor antagonism may be one of several co-existing therapeutic mechanisms, one or more of which may contribute to the clinical effectiveness of

that drug (eg. the anti-Parkinson's agent, memantine (Tomitaka et al., 1996), or the anticonvulsant agent, felbamate (Rho et al., 1994)).

Anticonvulsants could have significant relevance during warfare nerve-agent attacks, because anticonvulsants are administered to treat the seizures that can develop after exposure. The importance of treating soman-induced seizures was highlighted in a study by Filliat et al., (1999), who determined that soman-exposed rats were less likely to have hippocampal damage and memory impairment if they were spared from soman-induced seizures. Although most commonly used anticonvulsants are not thought to possess significant NMDA antagonist activity, several newly developed anticonvulsant agents may have sufficient activity at NMDA receptors to contribute to the therapeutic action. One such agent is felbamate, a highly effective anticonvulsant, not widely used because of serious hepatic and hematological adverse effects. However, it was such an effective anticonvulsant, that there is no doubt that interest will focus on developing other agents with a similar pharmacological profile, including activity as an NMDA receptor antagonist.

Other NMDA receptor antagonists that may be encountered by both civilian and military personnel include: antitussive agents such as dextromethorphan (in cough syrups), memantine (for treatment of Alzheimer's disease, or neuropathic pain), and alcohol- which is another commonly encountered agent with NMDA receptor antagonist properties. Alcohol is used widely across our society for recreational purposes, sometimes it is used to relieve stress, and it is often abused. Alcohol can potentiate GABA responses in some areas of the brain, and thus, might be neuroprotective, but this effect does not occur throughout the brain and may leave some brain regions vulnerable.

Olney and colleagues, as well as other investigators observed behavioral changes in animals to which NMDA receptor antagonists have been given (Olney et al., 1991, Loscher and Honack, 1991, Honack and Loscher, 1993). Often, these behaviors occur in animals that have vacuolization or other evidence of neurotoxicity, however, this is not always the case, as glycine-site antagonists produce a range of behavioral side effects not related to neurotoxicity (Berger et al., 1994).

Since we hypothesized that the addition of AChEI agents would exacerbate the neurotoxic effects of NMDA receptor antagonists, we took an *in vivo* approach to this problem to determine the following: whether the behavioral effects of NMDA receptor antagonist-mediated

neurotoxicity are modulated by co-exposure to AChEI agents, whether the histopathological effects of NMDA receptor antagonist-mediated neurotoxicity are modulated by co-exposure to AChEI agents, and finally, whether behavioral changes induced in the animals by either class of agents or by co-exposure to these agents reflect the animal's histopathological results. This is a standard approach for neurotoxicity screening batteries, the purpose of which is to use a functional observation battery (FOB), and assessments of motor activity, and neuropathology to screen potentially neurotoxic agents. The functional observation battery is noninvasive, and is used to determine any gross functional deficits in exposed animals. The motor activity assessments measure the level of activity for exposed animals. The addition of histopathological data allows the experimenter to characterize any concordance between functional neurological and neuropathological effects (EPA, 1998).

I: <u>Behavioral analysis:</u>

Analysis of NMDA receptor antagonist interactions with the AChEI agents: pyridostigmine bromide and physostigmine using a functional observation battery.

Katherine H Jones, Suzanne Clark, Elena Kuhn, and Wilkie A Wilson

Introduction

Neurotoxic screening of animals is of primary importance in the risk assessment of chemical agents and drugs. The process of neurotoxic screening includes: hazard identification (eg. to determine whether an agent increases the incidence of neurotoxicity), dose-response assessment, exposure assessment and risk assessment (Tilson and Moser, 1992). Behavioral indicators of neurotoxicity can detect drug-induced changes in nervous system activity that reflect changes in integrated sensory, motor, and cognitive function. Drug-induced behavioral changes often precede neuropathological signs of toxicity. Behavioral data have proved valuable for Federal agencies such as the Environmental Protection Agency (EPA) to set threshold limits for exposure to hundreds of chemicals, especially in the workplace. Thus, the EPA has established guidelines to follow in screening animals for neurotoxicity, which includes the use of a functional observation battery (FOB), an assessment of motor activity, and an assessment of neuropathology.

Based on the 'Health Effects Test Guidelines' (EPA, 1998), and work by Irwin (1968), and Tilson and Moser (1992), we designed a FOB to measure behavioral changes and changes in motor activity in the rat. The FOB is performed using a prescribed series of observations and tests that range from non-interactive to interactive assessments of each animals behavior and motor capabilities after they are exposed to the NMDA receptor antagonist or AChEI agent of interest (or a combination of these two classes of agents). The FOB we used was designed to assess several neurobiological domains: neuromuscular (weakness, incoordination, gait, posture, abnormal movements, tremor, seizures), sensory, and autonomic (Tilson and Moser, 1992).

Methods

Drugs

The drugs used in this study were from Sigma-Aldrich, St. Louis, MO: pyridostigmine bromide (PB): 3-(Dimethylaminocarbonyloxy)-1-methylpyridinium bromide, physostigmine hemisulfate (eserine hemisulfate), (+)-MK-801 hydrogen maleate: (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine, dextromethorphan hydrobromide monohydrate: d-3-Methoxy-N-methylmorphinan; felbamate: 2-phenyl-1,3-propanediol dicarbamate, and memantine: 3,5-dimethyladamantane. Paraformaldehyde, potassium permanganate, sodium chloride, dibasic sodium phosphate and monobasic sodium phosphate, were also purchased from Sigma-Aldrich, St. Louis, MO. Fluoro-Jade B (FJ-B) was purchased from Histo-Chem Inc., Jefferson, AR.

Animals

Results from previous studies show that female rats are more sensitive than male rats to NMDA receptor induced neuronal toxicity (Olney et al., 1989, Auer, 1996), we therefore only used adult female Sprague-Dawley retired breeder rats for this study. Retired breeder rats are typically 3-6 months old. Rats were ordered from Charles River (Raleigh, NC) and housed in groups of two-three animals in clear polycarbonate cages (27.9 X 27.9X 17.8 cm^3) on a 12 hr light/dark cycle at the Durham Veterans Affairs Medical Center (VAMC) Vivarium. All animal procedures were approved by the Durham VAMC Institutional Animal Care and Use Protocol. The VAMC is an AAALAC accredited institution. Three days after arrival, the animals were

individually weighed and randomly assigned to control or treatment groups. The average rat weight was 382.0g (+/- 8.8, SEM). Rats were given drugs or vehicle using intra-peritoneal (i.p.) or subcutaneous (s.c.) injections in a volume of 1.0ml/kg (in some cases, to ensure the drug was properly dissolved, drugs were given in a larger volume- eg. 2.0ml/kg was given to animals that received 50mg/kg dextromethorphan). Drugs were prepared fresh on injection day; PB, MK-801, dextromethorphan and memantine were each dissolved in sterile 0.9% saline. Felbamate was mixed in DMSO to 400 or 300mg/ml, was heated and stirred until it dissolved, and was finally injected at 1ml/kg. In each experiment, rats were first injected with the NMDA receptor antagonist of interest (MK-801, dextromethorphan, felbamate or memantine), followed within 15 minutes by injection with the AChEI agent, PB. We used the current literature to choose dose ranges for each drug; for the NMDA receptor antagonists, we based our doses on those used in rat neuroprotection studies. The low PB dose we used was roughly equivalent to that used clinically in humans to inhibit approximately 20% of peripheral cholinesterase (Servatius et al., 1998). The doses given for each drug were as follows: PB: 0.1, 0.3 and 1.0mg/kg, physostigmine: 0.1 and 0.3mg/kg, (+)MK-801: 0.3mg/kg, dextromethorphan: 10, 20 and 50mg/kg, felbamate: 100, 300 and 400mg/kg, and memantine: 25, 37.5, 50 and 75mg/kg. Control animals were given 0.9% saline (s.c.) followed by a second 0.9% saline injection (i.p.).

Functional observation battery (FOB)

The design and measurements of our functional observation battery (FOB) were based on observation batteries designed by others; specifically, to distinguish the different effects of neurotoxicants by observing various neurobehavioral changes in rodents (Irwin 1968, Moser 1990, 1996, Gad 1982, Tilson and Moser, 1992, United States E.P.A., 1998). The behavioral tests were administered to each animal prior to drug exposure (pre-drug), and additionally at one, four, twenty-four and forty-eight hours post-drug exposure by an observer that was blind to the treatment of each animal. The observer began the test panel (Table 1) by first observing each rat in its home-cage environment, then by observing each animal after it was removed to another cage for observation and manipulation (the arena).

Each home-cage observation was performed in the same sequence, beginning with an unobtrusive assessment of the animal's body posture, bizarre behavior, tremors, twitches, tonic or clonic convulsions, exophthalmus, and eye-crustiness. Body posture was rated on a scale of

1-10, with normal alert behavior scored at 1, and 10 describing a rat that is lying completely flat. Any bizarre behavior observed was given a score between 1-3 based on the degree of severity with a three as most severe. A brief description of the bizarre behavior was also noted. Both head-weaving and aimless wandering were typical bizarre behaviors that were observed. Tremors and twitches were also both scored (1-5); tremors were further described as exertion, head or tail tremors. Severe tremors or twitching rated higher scores. Convulsions were rated as either clonic or tonic and scored descriptively within those categories (between 1-3 for clonic convulsions and 1-5 for tonic convulsions). For example, clonic chewing behavior (scored as a one) is described as less severe than repetitive whole-body clonic tremors (scored as a three), and tonic opisthotonus (scored as a two) was described as less severe than popcorn convulsions (scored as a four). Finally, the presence of either exophthalmus or eye-crustiness was scored with a one (for present) or a zero (for absent).

After completion of the home-cage observations, the observer removed each rat to weigh (once each day of the study), then to a fresh cage (the field) to record the following: the animals state of arousal when removed from the home-cage (scored between 1-6, with a limp rat showing an absence of normal resistance scored as a 'one', a rat that displays some struggle but is easy to handle would be scored as a 'three' and a rat that is aggressive or otherwise difficult to remove from it's home-cage would be scored as a 'six'). In general, moderate scores describe a normal rat behavior. The presence of convulsions or tremors as a result of being handled, and the degree of palpebral closure were all scored as described above. The latency (seconds) before the animal's first step, whether or nor the animal exhibited piloerection, and general fur appearance were all recorded, as well as the total number of rears, grooming episodes, and the degree of tail elevation. The observer then manipulated the rat to assess: the degree of startle response elicited by a single clap (1-3, a score of two is a normal response, a score of one is no response), provoked biting elicited by placing a pencil in the rat's mouth (scored 1-5), the rat's reaction to the presence then approach of the observer's finger near the animal's head (also scored 1-5), and the rat's reaction to touch on the hindquarters (scored 1-7, with a one describing a none-responsive rat, and a seven a rat that leaps away from the observer's touch. Moderate scores from these evoked behaviors describe normal responses. Finally, the rat's gait, total gait incapacity and the degree of the animal's limb rotation (from normal stance) are recorded. Gait scores range from 1-6, with a one describing normal gait; two, a slightly ataxic gait; and six, a rat

that cannot walk. Total gait incapacity describes the ability of the rat to move around despite any gait disability, gait incapacity scores range from 1-4 with a one describing normal locomotion, and a four a rat that is totally impaired and cannot walk. Limb rotation scores range from 1-5 with one describing a normal rat with no limb rotation to a five, which describes a rat with severe limb rotation.

The rats were then handled by the tail to assess their degree of positional passivity (or struggle during tail suspension), their visual placing, grip strength, and ability to grip a pencil in their forepaws. Each of these behaviors was scored by degree of severity between 1-5, (or 1-3 for the pencil-grip test). Moderate scores described normal rat behaviors. Visual placing was assessed by holding the rat suspended above the field floor and then lowering the rat to the field floor, while observing the rat's forearm extension. A normal rat was scored with a value of four and displayed forearm extension well before the animal's vibrissae contacted the cage floor. Grip strength was scored as the animal's ability to grasp and hold onto the edge of the arena cage. The pencil grip tested the animal's ability to grasp a pencil placed in front of the rat- as the rat was suspended by the tail above the cage floor. The presence of hypothermia or lacrimation was recorded with a value of one. Each animal was tested for extensor thrust by holding the rat by the tail close to the arena cage bottom, and placing a hand on the pads of the animal's rear feet. Scoring ranged between 1-4. An unimpaired rat exhibits a strong push of the hind limbs against the observer's hand, and would thus be given a score of one. The non-responding, impaired rat would receive a score of four.

Pain responses were determined by quickly pinching one of the rat's hind limb toes, and their tails with blunt forceps and recording the response. The toe-pinch was scored between 1-5, and the tail-pinch between 1-7. Higher scores describe animals that are sensitive to either stimulation.

Righting reflexes were determined by flipping the animal onto it's back and recording the degree of impairment exhibited by the animal in righting itself. Similarly, the catalepsy score was determined by placing the animal's hindquarters onto a flat box 1.5 inches high in the arena cage, and recording the animal's ability to remove itself from that position. Both righting reflex and catalepsy scores range between 1-4, with a one describing the unimpaired rat, and a four a very impaired rat.

Finally, the presence of diarrhea and the degree of salivation were recorded; the animal's degree of irritability in being handled, and it's tendency to freeze were also scored. The total number of vocalizations, and the number of fecal boluses deposited in the arena cage within the three-minute test period were recorded at the conclusion of the functional observation battery.

Statistical analysis

Behavioral data was collected onto a spreadsheet by the observer, and then transferred into an Excel workbook, which was constructed to re-organize the data into specific functional domains. For quantitative evaluation, the behavioral scores determined at each of the four post-treatment time-points were summed for each rat and mean group values were calculated to allow comparisons between groups. Origin was used to calculate the means and standard error of the means (SEM) for each treatment group for each functional domain, and to graphically present the data. An additional one-way ANOVA was performed on the summed raw data using SPSS, with a Tukey HSD post-hoc analysis where necessary. Behavioral data for felbamate and felbamate-PB co-exposed animals was analyzed using an independent samples t-test.

Results

We grouped the 44 individual behaviors that were measured using the FOB (table 1) into twelve functional domains (table 2) that describe broad neurological functions; this was done to facilitate data analysis and interpretation of the data. The behavioral measurements were initially grouped into seven functional domains: neuromuscular, sensorimotor, CNS excitability, CNS activity, autonomic, general behavior, and motor affective. We then elected to further break down some functional domains into individual behaviors, resulting in a total of twelve 'domains' in our analyses. Thus, 'pain' was measured independently from other measurements in the sensorimotor domain, 'rearing' behavior was measured independently from other measurements in the CNS activity domain, gastrointestinal and secretory measurements were measured independently from other measurements in the autonomic domain, and both 'bizarre' behavior and 'positional passivity' were measured independently from other measurements in the general behavior domain.

Only the autonomic GI (gastrointestinal) domain remained unchanged by any treatment group. This was a cumulative effect, as animals that were exposed to AChEI agents typically

had several stools during the first hours post-drug injection; these animals then had fewer stools at the 24 and 48 hour time points post-drug injection, allowing the other treatment groups to 'catch up' and obfuscate any drug-induced effect.

Animals exposed to NMDA receptor antagonists exhibited the most significant behavioral effects. Only dextromethorphan (50mg/kg, n=2) had no significant effects on any of the monitored behaviors.

Several functional domains were only affected by MK-801; when compared to saline-injected control animals (n=12), 0.3mg/kg MK-801 (n=18) treated animals showed significant differences in the following functional domains: CNS activity ($p<0.000$, figure 1), general behavior ($p<0.003$, figure 2), motor affective behaviors ($p<0.003$, figure 3), pain ($p<0.004$, figure 4) and rearing ($p<0.031$, figure 5).

The autonomic functional domain was affected by agents in addition to MK-801 (figure 6A); when compared to saline injected control animals (n=12), 0.3mg/kg MK-801 (n=18) increased the deficit in the autonomic functional domain ($p<0.001$), 75mg/kg memantine also significantly increased this score ($p<0.006$, n=3) as did 400mg/kg felbamate ($p<0.05$, n=2).

Both 0.3mg/kg MK-801 ($p<0.000$, n=18) and 75mg/kg memantine ($p<0.001$, n=3) significantly reduced scores for positional passivity, as shown in figure 7.

The score for bizarre behavior significantly increased in animals exposed to 0.3mg/kg MK-801 ($p<0.027$, n=18, figure 8A).

The score for behaviors in the neuromuscular domain were significantly increased for animals exposed to 75mg/kg memantine (figure 9A): a 75mg/kg (n=3) dose ($p<0.024$) significantly increased this score when compared to control animals.

In contrast to the NMDA receptor antagonists, animals exposed to a low dose of the AChEI, PB (0.3 mg/kg, n=4) experienced a significant increase in the CNS/excitability score ($p<0.024$, figure 10). This was the only functional domain affected by PB exposure alone. Interestingly, all four animals dosed with 37.5mg/kg memantine had the same score, thus presenting no standard error of the means.

When combined with (0.3mg/kg) MK-801, PB exacerbated the effect of MK-801 in several functional domains. Compared to animals exposed to MK-801 alone, the score in the autonomic functional domain increased, ($p<0.011$, figure 6B) as did the score for bizarre behavior ($p<0.001$, figure 8B). PB also increased scores in the neuromuscular domain in MK-

801 co-exposed animals (figure 9B); A 0.3mg/kg PB dose significantly increases this score ($p<0.001$), as does 1.0mg/kg ($p<0.000$) in co-exposed animals, (although scores in the neuromuscular functional domain were not significantly changed in MK-801-exposed animals when compared to control animals). PB significantly modulates scores in the CNS excitability domain (figure 10) in animals co-exposed to 1.0mg/kg PB and 0.3mg/kg MK-801 ($p<0.047$), when compared to MK-801-exposed animals (using a post-hoc Tukey HSD test).

PB and MK-801 significantly modulated the sensorimotor functional domain in co-exposed animals (figure 11). As shown, 0.3mg/kg MK-801 (n=18) has a modest effect on the sensorimotor functional domain score, and 1.0mg/kg PB has no effect (n=8), however, the combination of the two agents in co-exposed animals (0.1mg/kg PB) decreases the sensorimotor score significantly ($p<0.001$, n=7).

The presence of physostigmine significantly exacerbated scores in several domains, although by itself, it did not affect scores in any functional domains. When compared to animals that received 25mg/kg memantine alone (n=5), the AChEI, physostigmine (0.3mg/kg) exacerbated the autonomic functional domain score in animals co-exposed to 25mg/kg memantine (figure 6C) ($p<0.031$, n=3). Interestingly, physostigmine (0.3mg/kg) ameliorated the effects of 0.3mg/kg MK-801 in co-exposed animals when compared to animals exposed only to MK-801 (figure 9B) ($p<0.009$, n=8).

Discussion

The behavioral effects of NMDA receptor antagonists have been investigated by several labs, which found distinct pharmacological profiles depending on whether they were investigating competitive or non-competitive receptor antagonists. Non-competitive NMDA receptor antagonists typically exert stimulatory effects on the behavior (eg., increased locomotion, stereotyped sniffing), and competitive antagonists do not typically induce stimulatory behaviors (reveiwed by Bubser et al., 1992). Most of the significant behavioral changes we found were induced by a low dose of MK-801. However, we also found behavioral changes in high-dose memantine exposed animals, that reflect the stereotypical behaviors induced by non-competitive NMDA receptor antagonists (Bubser et al., 1992).

Changes in the CNS activity domain reflect observable changes in the behavior of the animal prior to any interaction with the observer. The observer quietly assessed each of three

differently colored rats in their home-cage environment for their body posture, and the presence of tremors, twitches or convulsions. Body posture describes a range of positions: from sitting upright- alert, to lying prone. A higher score describes a more debilitated animal. In these experiments, of the NMDA receptor antagonists, only MK-801 produced a significantly debilitated animal, however, a 75mg/kg dose of memantine was also debilitating (although not significantly so). The presence of AChEI agents PB and physostigmine did not themselves significantly affect the score in the CNS activity domain, nor did they appear to significantly exacerbate the effect of MK-801.

General behavior domain scores were only significantly different in animals exposed to MK-801. Scores for the general behavior domain were determined by the observer after the animal has been removed from the home-cage to the field cage. These observations do not rely on interactions between the animal and observer beyond the animals transfer, but include: palpebral closure, fur appearance, grooming behavior and provoked biting. Based on our observations, palpebral closure was the primary score within this domain, as the animals were only occasionally dirty or disheveled, were rarely provoked to bite, and rarely groomed themselves in the field.

Motor-affective behaviors were also only significantly different in animals exposed to MK-801- however, a larger group number may have revealed a significant difference in animals exposed to 75mg/kg memantine or to 400mg/kg felbamate. Because of variability between animals, three animals exposed to 75mg/kg memantine and two animals exposed to 400mg/kg felbamate only describes a trend in this functional domain. Motor-affective behaviors were measured by the observer who interacted with the animal, and recorded each animal's response. The animals' state of arousal as they were removed from the home cage to the field was recorded, as were: the time (in minutes) for the animal to begin to move about, the animals irritability in being handled, the degree of freezing behavior elicited by a sound, and the total number of vocalizations made by the animal during the first three minutes in the field.

Pain was measured as an independent variable from the sensorimotor domain. Again, only MK-801 had a significant effect on pain. Pain was assessed by adding scores together from a tail-pinch and a toe-pinch. The toe-pinch tended to elicit a greater reaction from animals than did the tail-pinch. MK-801 reduced the animals' pain score, which one might have predicted, as NMDA receptor antagonists typically have analgesic properties. One might have also predicted

a reduced pain effect by the other tested NMDA receptor antagonists, however, instead, memantine at all doses had no effect on pain, and 400mg/kg felbamate appeared to increase the animals' sensation to pain.

Rearing behavior significantly decreased in MK-801 exposed animals, however, it appears that animals exposed to all doses of memantine or to felbamate also experienced decreased rearing behavior, and in fact, the three animals exposed to 37.5mg/kg memantine never reared- this is probably a statistical anomaly, but in general, MK-801 and memantine decreased rearing behavior. Rearing was measured by counting the total number of times an animal reared in the field during the first three minutes the animal was placed in the field. One might expect that rearing behavior may be reduced in animals experiencing ataxia or problems in locomotion. Or, an impaired animal may simply be less curious about it's immediate surroundings.

Autonomic behaviors were measured both in the home-cage (palpebral closure, exophthalmus and eye-crustiness) and in the field, where the degree of piloerection, hypothermia (a subjective measurement by the observers grasp on the animals' tails) and the degree of lacrimation were observed. MK-801, 75mg/kg memantine and felbamate all significantly increased scores in this domain. Based on our observations, these antagonists primarily increased scores in palpebral closure, exophthalmus and eye-crustiness. Palpebral closure was measured both in the home-cage and then after removal to the field; the palpebral closure score measured in the autonomic functional domain was a home-cage assessment. Often, the animals were piled together asleep when the observer quietly entered the room, and those that barely roused themselves, or remained asleep gained a higher score than those animals that became quickly alert and sat up. Although the AChEI agent, PB had no effect on autonomic function at any of the three tested doses, the addition of PB (at all three tested doses) significantly increased the autonomic function domain score in MK-801 co-exposed animals, (when compared to animals exposed only to MK-801). Thus, PB exacerbates the effect of MK-801 in the autonomic function domain. In contrast to MK-801, memantine appears to interact with physostigmine to modulate autonomic function domain scores; 25mg/kg memantine has no effect on autonomic function scores when given by itself, however, the presence of 0.3mg/kg physostigmine significantly increases this score.

The effect of NMDA receptor antagonists and AChEI agents on positional passivity was simply measured by the observer first grasping the animal by the tail and then holding the animal by the tail just above the field surface to observe and record the animals' struggle response. MK-801 and 75mg/kg memantine both similarly reduced the degree of struggle presented by the animal, and felbamate had a modest effect. Most animals were anxious to escape from the observer's grasp, and would reach and struggle to place her forepaws on the surface. Impaired animals often would not reach or struggle, and often even the animals' vibrissae making contact with the field surface failed to elicit reaching or struggle behaviors. Impaired animals usually recovered by 48 hours post injection, but often the MK-801 and 75mg/kg memantine-exposed animals showed residual impairment even at the 48-hour time point.

Bizarre behavior was measured separately from the general behavior category, primarily because many animals exhibited bizarre behaviors, and we did not want to obfuscate any significant differences with the general behavior functional domain. The bizarre behaviors were assessed by the observer both in the home-cage prior to any interaction with the animal and then again immediately after the animals' transfer to the field. An animal with a high bizarre behavior score probably exhibited the behavior in both environments. Most recorded bizarre behaviors were highly repetitive behaviors such as head-weaving, and aimless wandering; often once the animal is placed into the field arena, some of these behaviors subsided a bit. As expected, bizarre behaviors were greater in the hours immediately following exposure to MK-801, memantine and felbamate; and generally ceased by the 24-hour post-injection time-point. Exposure to MK-801 produced a significant increase in bizarre behaviors, however, animals co-exposed to MK-801 and PB experienced a significant increase in bizarre behavior over those only exposed to MK-801.

Neuromuscular functional domain behaviors primarily assessed the animals' muscular strength, tone, ability to ambulate, and the presence of Straub tail. All of the measurements in this domain were gathered by interacting with the animal in a prescribed sequence of events. The NMDA receptor antagonists: MK-801, and memantine increased the neuromuscular domain score, although dextromethorphan and felbamate both appeared to decrease this score. Our observations noted that often, post-injection, animals that demonstrated reduced grip strength in the hours immediately following injection had hyper-grip strength in later tests. The gait scores and the extensor thrust scores also typically improved dramatically as the drug effects wore off,

although often, limb rotation remained throughout 24 hours. It seemed with the pencil grip measurement, some animals were never able to hold onto the pencil while suspended by the tail approximately 12 inches above the field surface- they were too anxious to reach with their forelegs. On average, animals that were able to initially grip the pencil were not able to grip when impaired with MK-801 or memantine.

The CNS excitability functional domain measured two behaviors: convulsions and tremors. Both behaviors were initially assessed by the observer while the rat remained in the home-cage; the second measurement of convulsions and tremors was made after the animal was moved to the field. Since the behaviors in this report describe those from surviving animals, (those animals that expressed convulsions typically died, and were co-injected with NMDA receptor antagonists and PB) this measurement is primarily a measurement of tremors exhibited by the animal in the field. Because PB is an AChEI agent, it is not an unexpected result that animals exposed to PB may experience tremors. It is interesting that although MK-801 does not significantly increase the incidence of tremors, it does interact with the PB to significantly increase the tremor score over the score of animals exposed to MK-801 alone.

Sensorimotor domain behaviors included those that were elicited by the observer from the animal in the field. These included avoidance responses: startle response, response to an approaching finger, or a withdrawing finger, and the degree of escape elicited by touching the animal gently on the rear quarters with a pencil. We also measured: the animals' ability to visually place it as it was lowered by the tail to the field surface, the animals' righting reflex, and degree of catalepsy. Only the most impaired animals exhibited difficulty in righting itself, or would not move when placed with the back end of the animal was placed on a slightly raised platform.

As reviewed by Rogawski and Wenk (2003), channel blocking NMDA receptor antagonists fall into two main categories: disassociative anesthetic-like agents and low-affinity antagonists. At low doses, the behavioral effects of disassociative agents produce hyper locomotion, stereotypical behaviors, and ataxia. Higher doses produce immobility, analgesia and amnesia. The results from our FOB clearly indicate that as expected, a low dose of MK-801 does produce hyper locomotion behaviors, stereotypical (bizarre) behaviors, and ataxia. Ataxia is perhaps best measured in our FOB by examining neuromuscular domain behaviors (gait) and motor-affective behaviors (time to first movement). Animals co-exposed to PB and MK-801

tended to show exacerbated effects in these parameters. Thus, PB appears to interact with MK-801 to exacerbate the effects associated with this disassociative agent- or to perhaps increase NMDA receptor-mediated toxicity within the CNS.

The highest tested doses of memantine we used (37.5mg/kg, and 75mg/kg) increased hyper locomotion, bizarre behaviors, and ataxia in exposed animals. These results, including those with MK-801, are similar to those of Bubser et al., (1992), who found that 0.33mg/kg MK-801 and 20mg/kg memantine increased stereotypical behaviors such as head weaving and sniffing, and increased ataxia. Based on experiments by others that examined the behavioral effects of local infusions of NMDA receptor antagonists, Bubser et al., (1992) suggested changes in stereotypical behaviors reflect the activity specifically of non-competitive NMDA receptor antagonists, and also indicate that MK-801 and memantine may be affecting dopaminergic activity in the basal ganglia region of the brain.

One characteristic feature of the NMDA receptor antagonists we tested (with the exception of dextromethorphan) was the inhibition of rearing behavior; this result has been reported by others (Danyz et al., 1994), and may simply reflect an increase in ataxia and an inability to rear, or it may instead reflect a lack of interest in exploring the environment.

Most of the significant results we found in behavioral changes were induced by a low dose of MK-801 (0.3mg/kg); high doses of memantine are required to have significant effects in the autonomic behavior, positional passivity, and neuromuscular domain scores, and felbamate only significantly effected the autonomic function domain score. PB interacted with MK-801 and memantine to modulate behaviors in the autonomic function, bizarre behavior, neuromuscular, and sensorimotor domains. PB exposed animals experienced changes in the CNS excitability domain, and PB exacerbated this score in MK-801 co-exposed animals. To assess whether these changes in behavior reflect the resulting histopathology in the brain, animals exposed to the NMDA receptor antagonists: MK-801, dextromethorphan, felbamate and memantine, or exposed to the AChEI agents: PB and physostigmine, and animals co-exposed to these agents had their brains post-fixed for histopathological analysis 72 hours after drug exposure (to allow time for an excitotoxic response to develop).

Figure legends

Table 1: Functional observation battery test panel. The observer used a test panel to sequentially test and score behaviors of interest at each time-point pre and post-drug treatment. The observer first scored home cage observations (behaviors 1-8), followed by non-interactive, arena observations (behaviors 9-18). The scoring of the remaining behaviors relied increasingly on interactions with the observer, for example, positional passivity and extensor thrust observations.

Table 2: Functional behavioral domains. The results scored on the test panel were entered into a computer spreadsheet designed to re-organize the data into functional domains. This enabled us to score multiple behaviors for each domain in the least obtrusive manner possible. In this manner, 45 observed behaviors at each time-point were re-organized into 11 functional domains at each time-point for each animal (animals 1-8).

Figure 1: NMDA receptor antagonists: Effect on CNS activity. Animals were given the functional observation battery (FOB) pre-test 24 hours prior to drug exposure, and then were given a single s.c. injection of saline or the NMDA receptor antagonist of interest: 0.3mg/kg MK-801, 25, 37.5 or 75mg/kg memantine, 50mg/kg dextromethorphan, or 400mg/kg felbamate; followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. The CNS-activity domain scores were determined by adding together scores for each behavior within the CNS-activity domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: ** ($p<0.001$). Note that when compared to control animals, 0.3mg/kg MK-801 significantly increases the CNS-activity domain score, which is determined by in-home cage analysis (see text) of the following parameters: body posture, tremors, twitches, and convulsions. A high dose of memantine (75mg/kg) also appears to increase the CNS activity score.

Figure 2: NMDA receptor antagonists: Effect on general behavior. Animals were given the FOB pre-test 24 hours prior to drug exposure, and then were given a single s.c. injection of saline or the NMDA receptor antagonist of interest: 0.3mg/kg MK-801, 25, 37.5 or 75mg/kg

memantine, 50mg/kg dextromethorphan, or 400mg/kg felbamate; followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. The general behavior domain scores were determined by adding together scores for each behavior within the general behavior domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: ** ($p<0.001$). Note that when compared to control animals, 0.3mg/kg MK-801 significantly decreases the general behavior domain score. The general behavior domain is a non-interactive, field assessment of: palpebral closure, fur appearance, grooming activity and provoked biting.

Figure 3: NMDA receptor antagonists: Effect on motor-affective behavior. Animals were given the FOB pre-test 24 hours prior to drug exposure, and then were given a single s.c. injection of saline or the NMDA receptor antagonist of interest: 0.3mg/kg MK-801, 25, 37.5 or 75mg/kg memantine, 50mg/kg dextromethorphan, or 400mg/kg felbamate; followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. The motor-affective domain scores were determined by adding together scores for each behavior within the motor-affective domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: ** ($p<0.001$).
Note that when compared to control animals, 0.3mg/kg MK-801 significantly increases the score for the motor-affective domain, which is an interactive assessment of: the animals state of arousal (when moved from the home-cage to the field), the time to first movement after being placed in the field, grasp irritability, provoked freezing, and the total number of vocalizations made by the animal during the first three minutes in the field. Also, note that 75mg/kg memantine appears to increase the motor-affective score.

Figure 4: NMDA receptor antagonists: Effect on pain sensation. Animals were given the FOB pre-test 24 hours prior to drug exposure, and then were given a single s.c. injection of saline or the NMDA receptor antagonist of interest: 0.3mg/kg MK-801, 25, 37.5 or 75mg/kg memantine, 50mg/kg dextromethorphan, or 400mg/kg felbamate; followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours

and 48 hours post-injection. Pain scores were determined by adding together scores for toe and tail pinches (see text) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: ** ($p<0.001$). Note that when compared to control animals, 0.3mg/kg MK-801 significantly reduces the ability of animals to feel pain, however, felbamate appears to increase the animals' pain sensation.

Figure 5: NMDA receptor antagonists: Effect on rearing behavior. Animals were given the FOB pre-test 24 hours prior to drug exposure, and then were given a single s.c. injection of saline or the NMDA receptor antagonist of interest: 0.3mg/kg MK-801, 25, 37.5 or 75mg/kg memantine, 50mg/kg dextromethorphan, or 400mg/kg felbamate; followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. Rearing scores were determined by adding together the total number of "rears" an animal made during the first three minutes in the field (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: * ($p<0.01$). Note that when compared to control animals 0.3mg/kg MK-801 significantly decreases the rearing score. Also, memantine and felbamate appear to reduce rearing behavior. Curiously, animals exposed to 37.5mg/kg memantine never reared after drug exposure.

Figure 6A: NMDA receptor antagonists: Effect on autonomic behavior. Animals were given the FOB pre-test 24 hours prior to drug exposure, and then were given a single s.c. injection of saline or the NMDA receptor antagonist of interest: 0.3mg/kg MK-801, 25, 37.5 or 75mg/kg memantine, 50mg/kg dextromethorphan, or 400mg/kg felbamate; followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. The autonomic behavior domain scores were determined by adding together scores for each behavior within the autonomic behavior domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: * ($p<0.01$) and ** ($p<0.001$). Note that the autonomic behavior scores are significantly exacerbated by 0.3mg/kg MK-801, 75mg/kg memantine, and 400mg/kg felbamate when compared to control animals. The score for the

autonomic behavior domain is determined by assessing palpebral closure (in the home-cage), exophthalmus, eye-crustiness, piloerection, hypothermia, and lacrimation.

Figure 6B: MK-801 +/- AChEIs: Effect on autonomic behavior. Animals were given the FOB pre-test 24 hours prior to drug exposure, and then were given: a single s.c. injection of saline or 0.3mg/kg MK-801; followed by a single i.p. injection of saline, 0.1, 0.3 or 1.0mg/kg PB, or 0.1, 0.3mg/kg physostigmine. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. The autonomic behavior domain scores were determined by adding together scores for each behavior within the autonomic behavior domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: ## ($p<0.001$), and reflects a comparison to MK-801-saline injected animals (determined by a post-hoc Tukey HSD after running a one-way ANOVA). Note that the autonomic behavior scores are significantly exacerbated by the addition of PB at all tested doses. The score for the autonomic behavior domain is determined by assessing palpebral closure (in the home-cage), exophthalmus, eye-crustiness, piloerection, hypothermia, and lacrimation.

Figure 6C: 25mg/kg memantine +/- 0.3mg/kg physostigmine: Effect on autonomic behavior. Animals were given the functional observation battery (FOB) pre-test 24 hours prior to drug exposure, and then were given: a single s.c. injection of saline or 25mg/kg memantine; followed by a single i.p. injection of saline, or 0.3mg/kg physostigmine. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. The autonomic behavior domain scores were determined by adding together scores for each behavior within the autonomic behavior domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: ** ($p<0.001$). Note that autonomic behavior scores are significantly exacerbated by co-exposing animals to 25mg/kg memantine and 0.3mg/kg physostigmine. The score for the autonomic behavior domain is determined by assessing palpebral closure (in the home-cage), exophthalmus, eye-crustiness, piloerection, hypothermia, and lacrimation.

Figure 7: NMDA receptor antagonists: Effect on positional passivity. Animals were given the functional observation battery (FOB) pre-test 24 hours prior to drug exposure, and then were given a single s.c. injection of saline or the NMDA receptor antagonist of interest: 0.3mg/kg MK-801, 25, 37.5 or 75mg/kg memantine, 50mg/kg dextromethorphan, or 400mg/kg felbamate; followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. Positional passivity scores were determined by adding together the scores determined at each post-injection time-point (see text and table 2). Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: * ($p<0.01$) and ** ($p<0.001$) Note that when compared to control animals, 0.3mg/kg MK-801 and 75mg/kg memantine significantly decreases the positional passivity score; felbamate also appears to reduce this score. A decreased positional passivity score describes reduced struggle behavior as the animal was held by the tail 8-12 inches above the field surface.

Figure 8A: NMDA receptor antagonists: Effect on bizarre behavior. Animals were given the functional observation battery (FOB) pre-test 24 hours prior to drug exposure, and then were given a single s.c. injection of saline or the NMDA receptor antagonist of interest: 0.3mg/kg MK-801, 25, 37.5 or 75mg/kg memantine, 50mg/kg dextromethorphan, or 400mg/kg felbamate; followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. Bizarre behavior domain scores were determined by adding together scores for each behavior within the bizarre behavior domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: * ($p<0.01$).
Note that when compared to control animals, 0.3mg/kg MK-801 significantly increases the score for the bizarre behavior domain, which is an additive assessment of the animals' behavior both in the home-cage and in the field. Memantine and felbamate exposure appear to also increase the degree of bizarre behavior expressed by the animals.

Figure 8B: 0.3mg/kg MK-801 +/- PB: Effect on bizarre behavior. Animals were given the functional observation battery (FOB) pre-test 24 hours prior to drug exposure, and then were given: a single s.c. injection of saline or 0.3mg/kg MK-801; followed by a single i.p. injection of

saline or 0.1, 0.3, 1.0mg/kg PB. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. Bizarre behavior domain scores were determined by adding together scores for each behavior within the bizarre behavior domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: + ($p<0.01$). Note that when compared to 0.3mg/kg MK-801 exposed animals, 1.0mg/kg PB significantly increases the score for the bizarre behavior domain in 0.3mg/kg MK-801 co-exposed animals. Bizarre behavior is measured as an additive assessment of the animals' behavior both in the home-cage and in the field.

Figure 9A: **NMDA receptor antagonists: Effect on the neuromuscular domain.** Animals were given the functional observation battery (FOB) pre-test 24 hours prior to drug exposure, and then were given a single s.c. injection of saline or the NMDA receptor antagonist of interest: 0.3mg/kg MK-801, 25, 37.5 or 75mg/kg memantine, 50mg/kg dextromethorphan, or 400mg/kg felbamate; followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. Neuromuscular domain scores were determined by adding together scores for each behavior within the neuromuscular domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: * ($p<0.01$). Note that the neuromuscular domain scores are significantly exacerbated by 37.5 and 75mg/kg memantine when compared to control animals. The score for the neuromuscular domain is determined by assessing: Straub tail, gait score, gait incapacity, limb rotation, grip strength, body tone, pencil grip, and extensor thrust. The determination of the neuromuscular domain score is highly interactive, and thus requires that the observer handle the animal for most measurements within the domain.

Figure 9B: MK-801 +/- AChEIs: Effect on the neuromuscular domain. Animals were given the functional observation battery (FOB) pre-test 24 hours prior to drug exposure, and then were given: a single s.c. injection of saline or 0.3mg/kg MK-801; followed by a single i.p. injection of saline, 0.1, 0.3 or 1.0mg/kg PB, or 0.1, 0.3mg/kg physostigmine. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. Neuromuscular domain

scores were determined by adding together scores for each behavior within the neuromuscular domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: ** ($p<0.001$)- reflecting a comparison to saline injected animals, or by: ## ($p<0.001$), reflecting a comparison to MK-801-saline injected animals (determined by a post-hoc Tukey HSD analyses after running a one-way ANOVA). Note that the neuromuscular domain scores are significantly exacerbated by the addition of PB at 0.3 and 1.0mg/kg, and are ameliorated by 0.3mg/kg physostigmine when compared to 0.3mg/kg MK-801 exposed animals. The score for the neuromuscular domain is determined by assessing Straub tail, gait score, gait incapacity, limb rotation, grip strength, body tone, pencil grip, and extensor thrust.

Figure 10: **0.3mg/kg MK-801 +/- PB: Effect on CNS excitability.** Animals were given the functional observation battery (FOB) pre-test 24 hours prior to drug exposure, and then were given: a single s.c. injection of saline or 0.3mg/kg MK-801; followed by a single i.p. injection of saline or 0.1, 0.3, 1.0mg/kg PB. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. CNS excitability scores were determined by adding together scores for each behavior within the CNS excitability domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: * ($p<0.01$)- reflecting a comparison to saline injected animals, or by + ($p<0.001$)- reflecting a comparison to MK-801-saline injected animals (determined by a post-hoc Tukey HSD analyses after running a one-way ANOVA). Note that when compared to control animals, exposure to 0.3mg/kg PB significantly increases the CNS excitability score, and also, that 0.3 and 1.0mg/kg PB exacerbates the CNS excitability score in 0.3mg/kg MK-801 co-exposed animals. CNS excitability is measured as assessment of convulsions and tremors expressed by the animal after removal from the home-cage to the field.

Figure 11: **0.3mg/kg MK-801 +/- PB: Effect on sensorimotor activity.** Animals were given the functional observation battery (FOB) pre-test 24 hours prior to drug exposure, and then were given: a single s.c. injection of saline or 0.3mg/kg MK-801; followed by a single i.p. injection of saline or 0.1, 0.3, 1.0mg/kg PB. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. Sensorimotor domain scores were determined by adding

together scores for each behavior within the sensorimotor domain (see text and table 2) using each post-injection time-point. Scores were averaged for each treatment group and are expressed as the mean (+/-SEM). Significance is noted by: * ($p<0.01$) and ** ($p<0.001$). Note that when compared to control animals, 0.1mg/kg PB significantly decreases the sensorimotor domain score in 0.3mg/kg MK-801 co-exposed animals. Sensorimotor domain scores are measured as an assessment of: avoidance responses (finger approach, finger withdrawal, and touch-escape), startle responses, visual placing, righting reflex, and catalepsy.

Table 1: Functional observation battery test panel

Experiment:			Post-drug time:			
Drug 1/route:			Date:			
Drug 2/route:			Examiner init:			
Species: Rat		Animal code:	1	2	3	4
Strain: Sprague-Dawley		Body weight (g):				
Sex: female		Volume injected:				
Age: retired breeder		Time admin:				

	measurement	Score	1	2	3	4
1	Body posture	scale 1-9				
2	Bizarre behavior	present = 1, nature				
3	Tremors I	scale 1-5, nature				
4	Twitches	scale 1-5				
5	Convulsions I	clonic/tonic, scale				
6	Palpebral closure I	scale 1-4				
7	Exophthalmus	present = 1				
8	Eye crustiness	present = 1				
9	Arousal	scale 1-6				
10	Convulsions II	clonic/tonic, scale				
11	Tremors II	scale 1-5, nature				
12	Time to movement	seconds				
13	Palpebral closure II	scale 1-4				
14	Piloerection	present = 1				
15	Fur appearance	scale 1-3				
16	Rearing/3 min.	number/3 min				
17	Grooming/3 min.	number/3 min				
18	Tail elevation	scale 1-5				
19	Startle response	scale 1-3				
20	Provoked biting	scale 1-5				
21	Finger approach	scale 1-5				
22	Finger withdrawal	scale 1-5				
23	Touch-escape	scale 1-7				
24	Gait	scale 1-6				

	measurement	Animal code:	1	2	3	4
25	Total gait incapacity	scale 1-4				
26	Limb rotation	scale 1-5, code				
27	Positional passivity	scale 1-5				
28	Visual placing	scale 1-5				
29	Grip strength	scale 1-5				
30	Body tone	scale 1-5				
31	Hypothermia	present = 1				
32	Lacrimation	present = 1				
33	Toe-pinch	scale 1-5				
34	Pencil Grip	scale 1-3				
35	Extensor thrust	scale 1-4				
36	Diarrhea	present = 1				
37	Salivation	scale 1-5				
38	Tail pinch	scale 1-7				
39	Righting reflex	scale 1-4				
40	Catalepsy	scale 1-4				
41	Grasp irritability	scale 1-4				
42	Provoked freezing	scale 1-4				
43	Vocalizations	number of/3 min.				
44	Urination/defecation	number of/3 min.				
45	Death	present +				

Table 2: Functional behavioral domains

Functional domain	Behavior
Neuromuscular	Tail elevation
	Gait score
	Gait incapacity
	Limb rotation
	Grip strength
	Body tone
	Pencil Grip
	Extensor thrust
Sensorimotor	Startle response
	Finger approach
	Finger withdrawal
	Touch-escape
	Visual placing
	Righting reflex
	Catalepsy
Pain	Toe-pinch
	Tail-pinch
CNS excitability	Convulsions II
	TremorsII
CNS activity	Body posture
	TremorsI
	Twitches
	Convulsions I
	Rearing

Functional domain	Behavior
Autonomic/general	Palpebral closure I
	Exophthalmus
	Eye crustiness
	Piloerection
	Hypothermia
	Lacrimation
Autonomic/G.I.	Diarrhea
	Salivation
	Defecation
Behavioral:	Bizarre behavior
	Palpebral closure II
	Fur appearance
	Grooming
	Provoked biting
	Positional passivity
Motor affective:	Arousal
	Time to movement
	Grasp irritability
	Provoked freezing
	Vocalizations

Figure 1

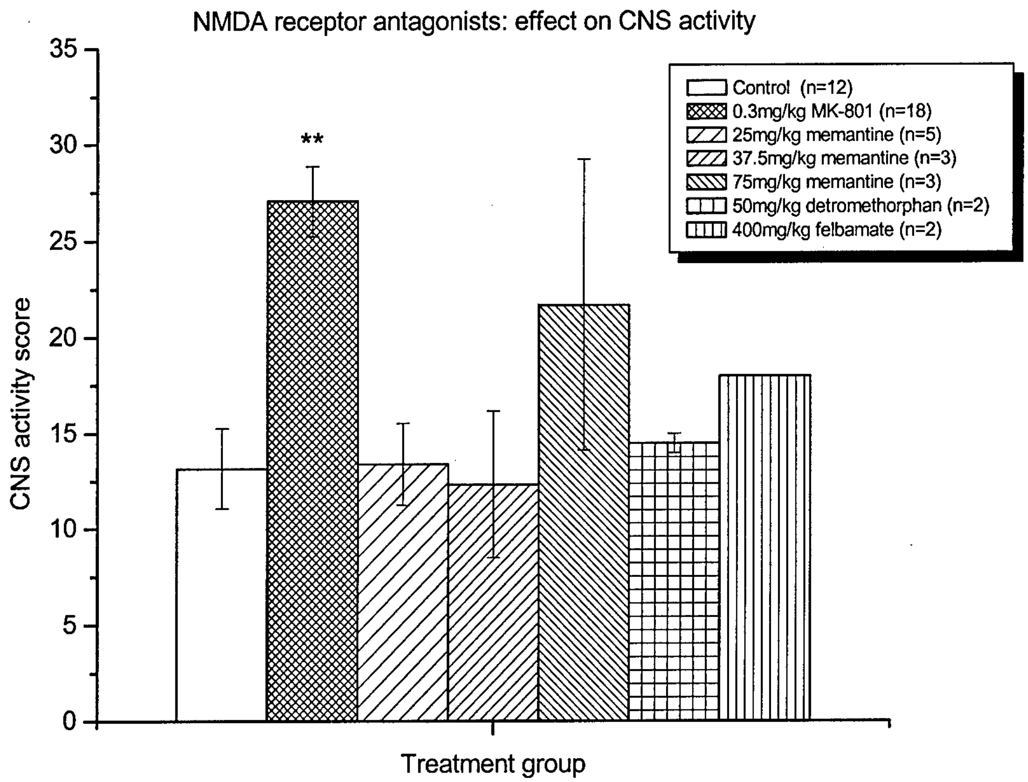

NMDA receptor antagonists: effect on CNS activity
Control (n=12)
0.3mg/kg MK-801 (n=18)
25mg/kg memantine (n=5)
37.5mg/kg memantine (n=3)
75mg/kg memantine (n=3)
50mg/kg detromethorphan (n=2)
400mg/kg felbamate (n=2)
**
35
30
25
20
15
10
5
0
CNS activity score
Treatment group

Figure 2

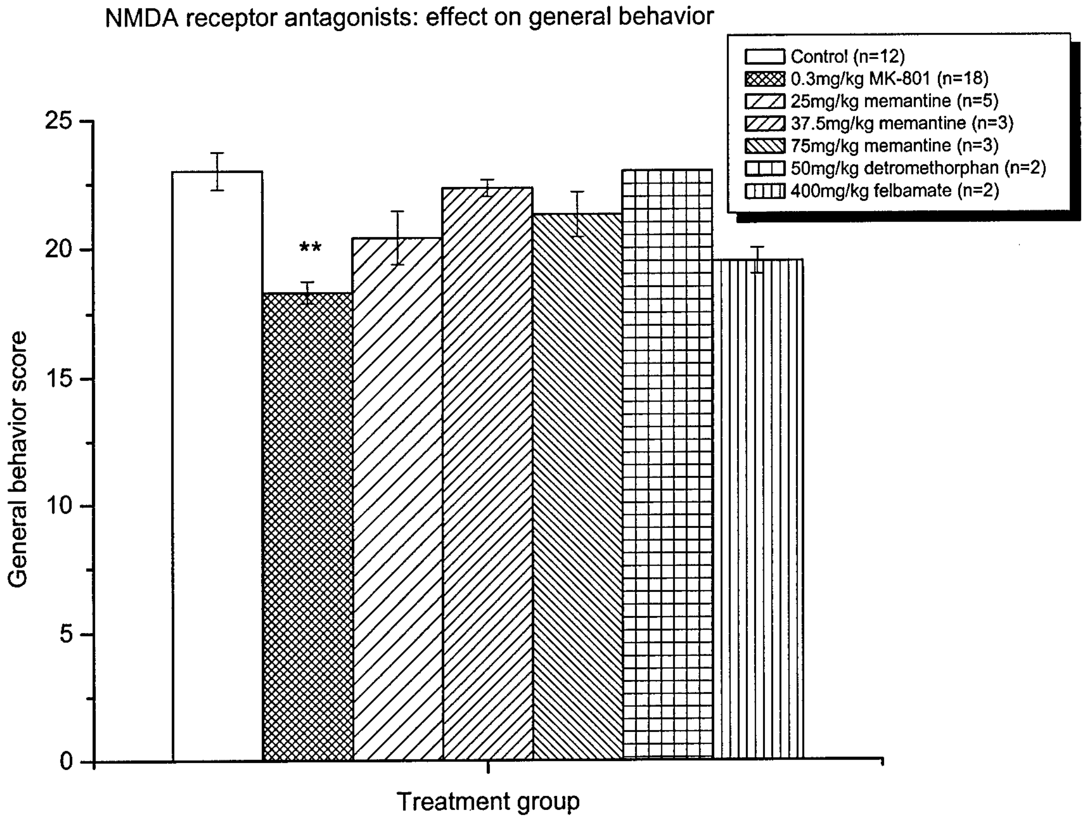

NMDA receptor antagonists: effect on general behavior
Control (n=12)
0.3mg/kg MK-801 (n=18)
25mg/kg memantine (n=5)
37.5mg/kg memantine (n=3)
75mg/kg memantine (n=3)
50mg/kg detromethorphan (n=2)
400mg/kg felbamate (n=2)
25
20
15
10
5
0
**
General behavior score
Treatment group

Figure 3

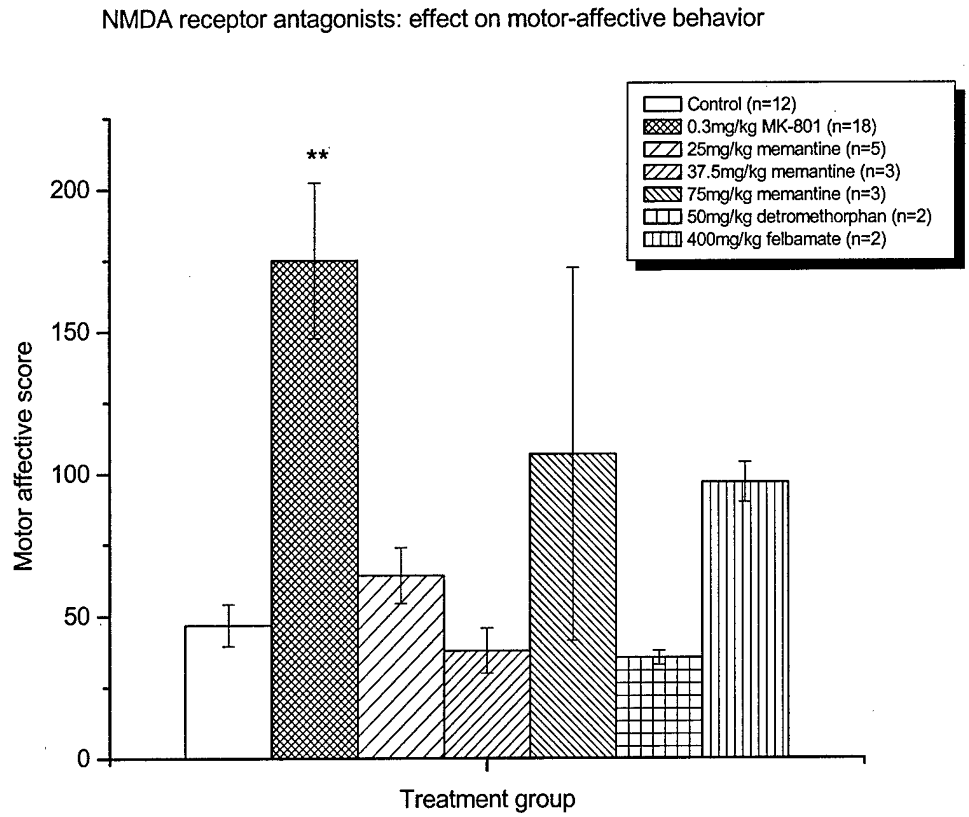
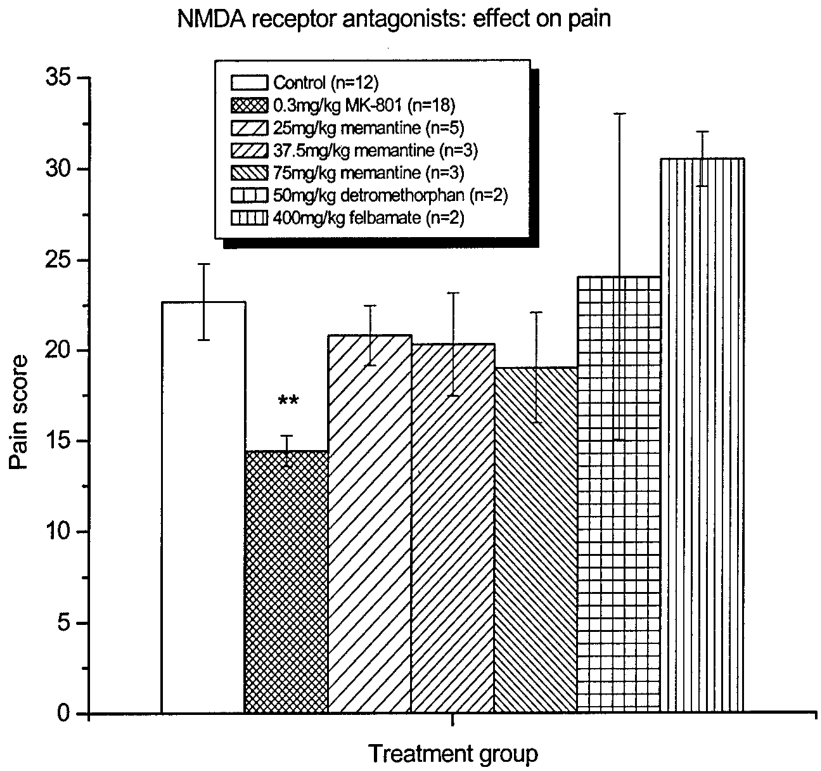

NMDA receptor antagonists: effect on motor-affective behavior
Control (n=12)
0.3mg/kg MK-801 (n=18)
25mg/kg memantine (n=5)
37.5mg/kg memantine (n=3)
75mg/kg memantine (n=3)
50mg/kg detromethorphan (n=2)
400mg/kg felbamate (n=2)
**
Motor affective score
0
50
100
150
200
Treatment group

Figure 4



NMDA receptor antagonists: effect on pain
Control (n=12)
0.3mg/kg MK-801 (n=18)
25mg/kg memantine (n=5)
37.5mg/kg memantine (n=3)
75mg/kg memantine (n=3)
50mg/kg detromethorphan (n=2)
400mg/kg felbamate (n=2)
**
Pain score
0
5
10
15
20
25
30
35
Treatment group

Figure 5

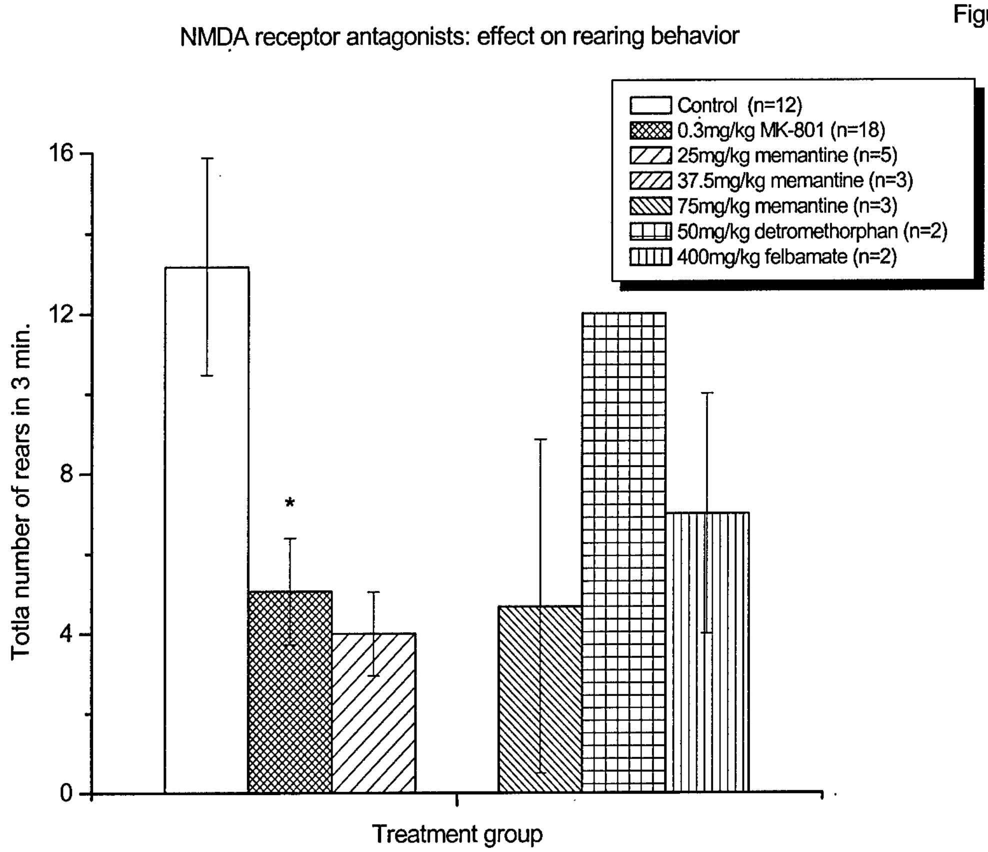
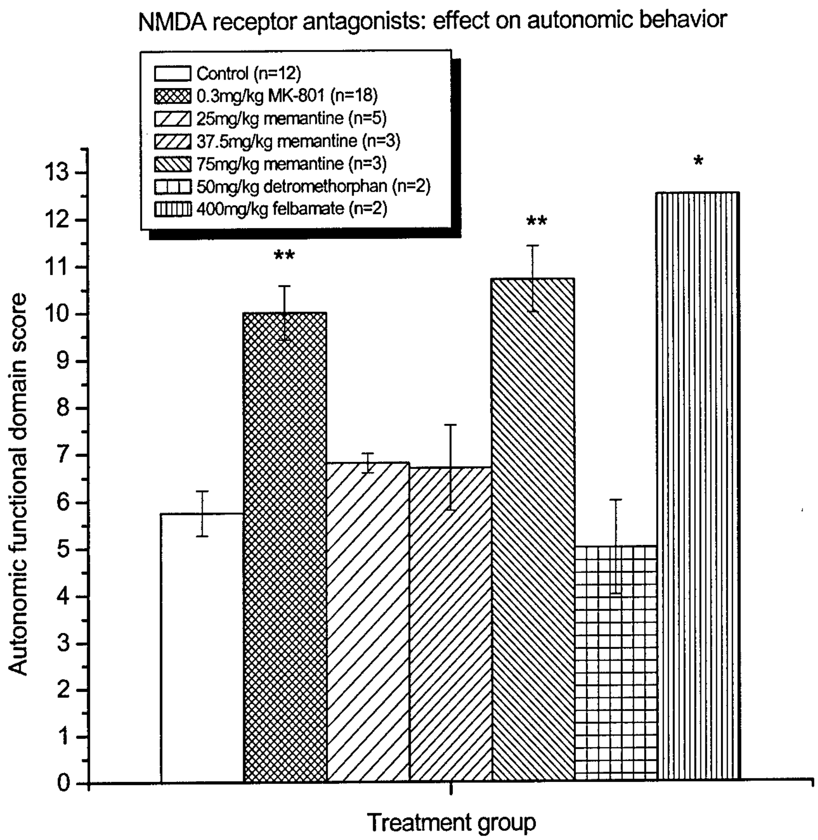

NMDA receptor antagonists: effect on rearing behavior
Control (n=12)
0.3mg/kg MK-801 (n=18)
25mg/kg memantine (n=5)
37.5mg/kg memantine (n=3)
75mg/kg memantine (n=3)
50mg/kg detromethorphan (n=2)
400mg/kg felbamate (n=2)
Totla number of rears in 3 min.
16
12
8
4
0
*
Treatment group

Figure 6A


NMDA receptor antagonists: effect on autonomic behavior
Control (n=12)
0.3mg/kg MK-801 (n=18)
25mg/kg memantine (n=5)
37.5mg/kg memantine (n=3)
75mg/kg memantine (n=3)
50mg/kg detromethorphan (n=2)
400mg/kg felbamate (n=2)
**
**
*
Autonomic functional domain score
0
1
2
3
4
5
6
7
8
9
10
11
12
13
Treatment group

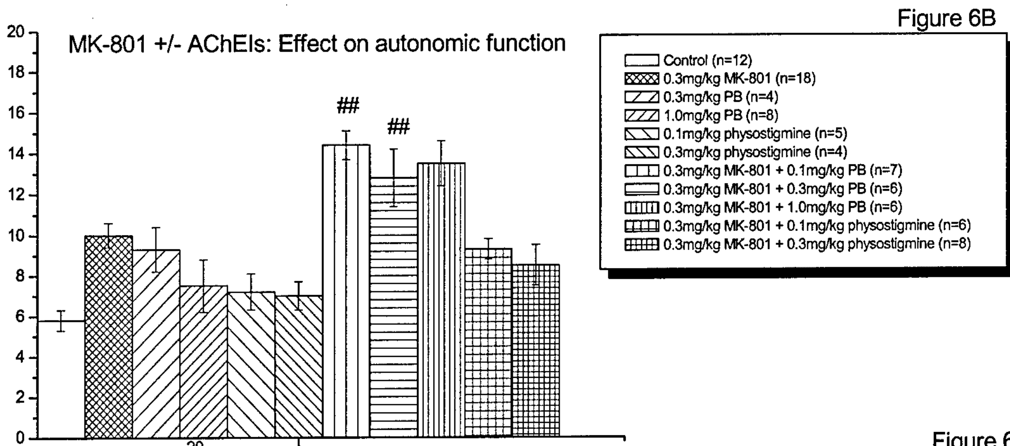


Figure 6B
MK-801 +/- AChEIs: Effect on autonomic function
Control (n=12)
0.3mg/kg MK-801 (n=18)
0.3mg/kg PB (n=4)
1.0mg/kg PB (n=8)
0.1mg/kg physostigmine (n=5)
0.3mg/kg physostigmine (n=4)
0.3mg/kg MK-801 + 0.1mg/kg PB (n=7)
0.3mg/kg MK-801 + 0.3mg/kg PB (n=6)
0.3mg/kg MK-801 + 1.0mg/kg PB (n=6)
0.3mg/kg MK-801 + 0.1mg/kg physostigmine (n=6)
0.3mg/kg MK-801 + 0.3mg/kg physostigmine (n=8)
##
##
0
2
4
6
8
10
12
14
16
18
20

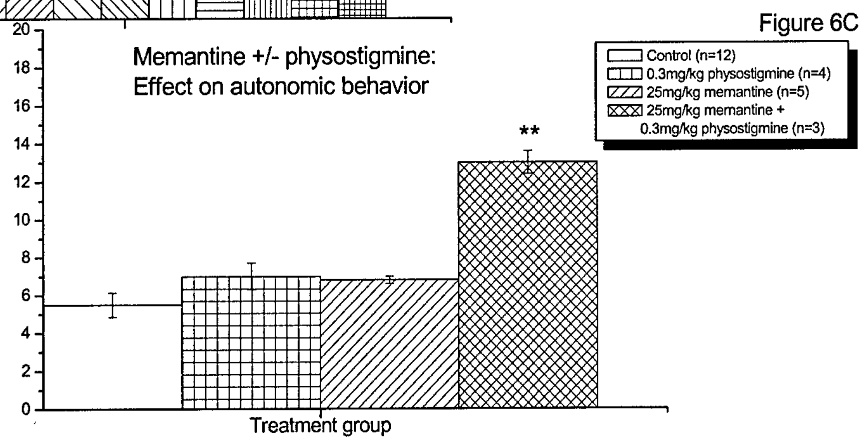
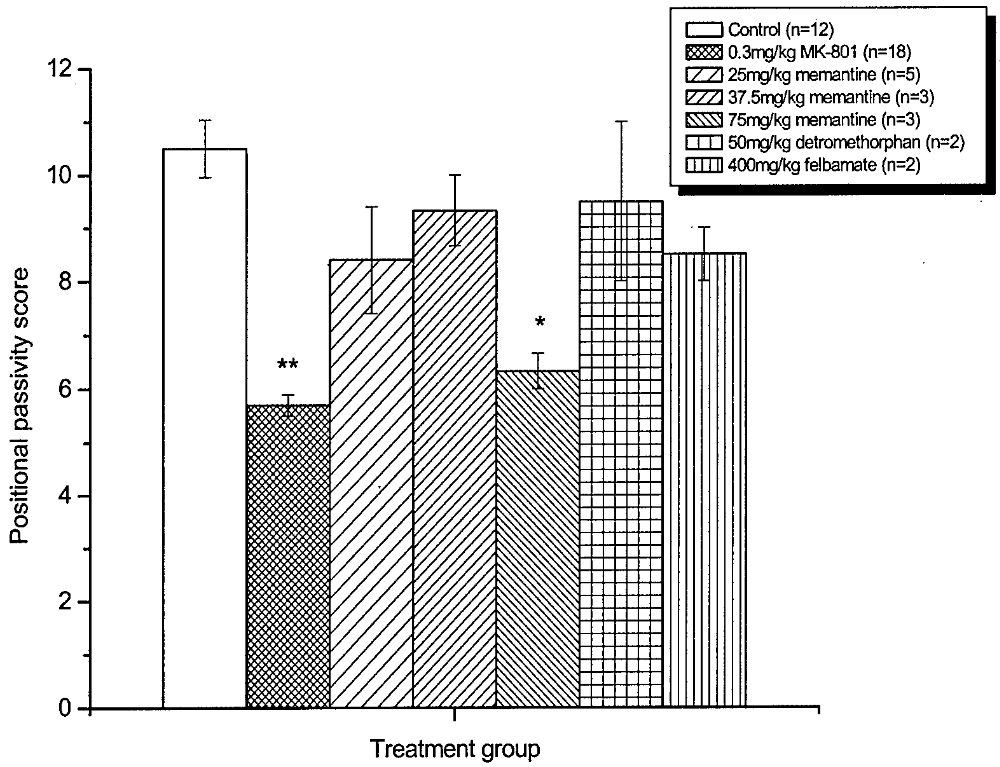


Figure 6C
Memantine +/- physostigmine:
Effect on autonomic behavior
Control (n=12)
0.3mg/kg physostigmine (n=4)
25mg/kg memantine (n=5)
25mg/kg memantine +
0.3mg/kg physostigmine (n=3)
**
0
2
4
6
8
10
12
14
16
18
20
Treatment group

Figure 7

NMDA receptor antagonists: effect on positional passivity



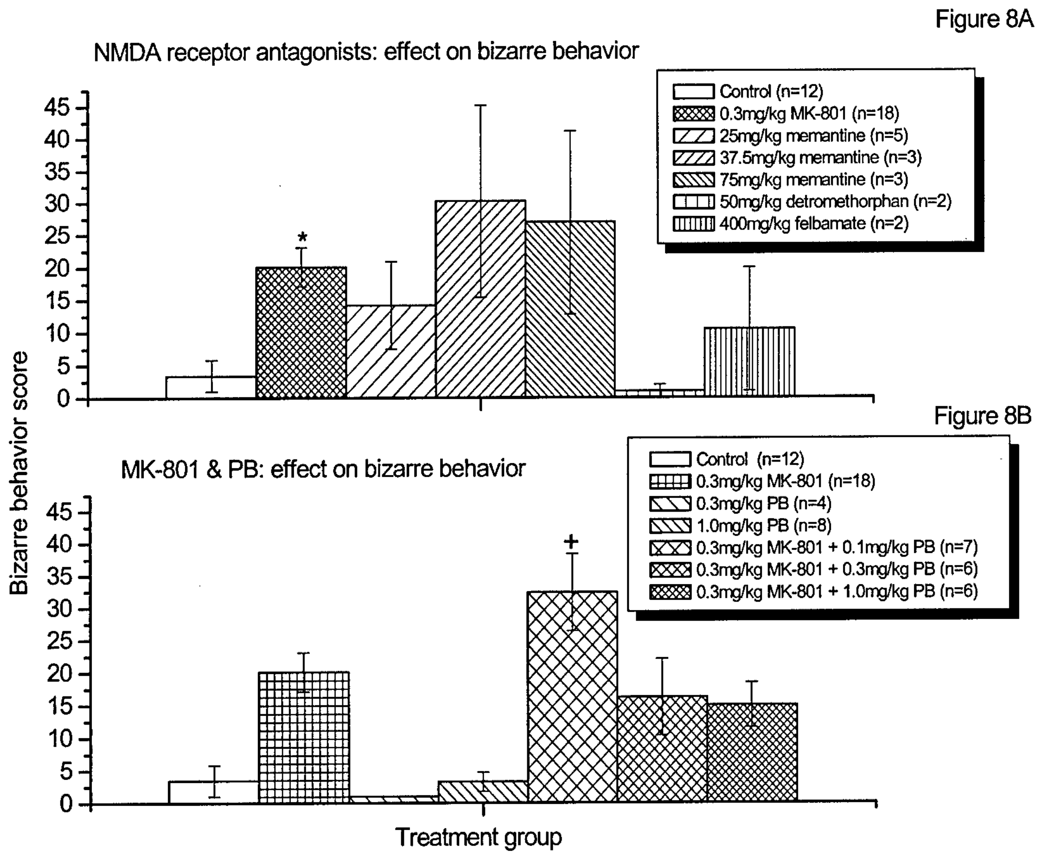


Figure 8A
NMDA receptor antagonists: effect on bizarre behavior
Control (n=12)
0.3mg/kg MK-801 (n=18)
25mg/kg memantine (n=5)
37.5mg/kg memantine (n=3)
75mg/kg memantine (n=3)
50mg/kg detromethorphan (n=2)
400mg/kg felbamate (n=2)
*
45
40
35
30
25
20
15
10
5
0
Figure 8B
MK-801 & PB: effect on bizarre behavior
Control (n=12)
0.3mg/kg MK-801 (n=18)
0.3mg/kg PB (n=4)
1.0mg/kg PB (n=8)
0.3mg/kg MK-801 + 0.1mg/kg PB (n=7)
0.3mg/kg MK-801 + 0.3mg/kg PB (n=6)
0.3mg/kg MK-801 + 1.0mg/kg PB (n=6)
+
Bizarre behavior score
Treatment group

Figure 9A

Figure 9B

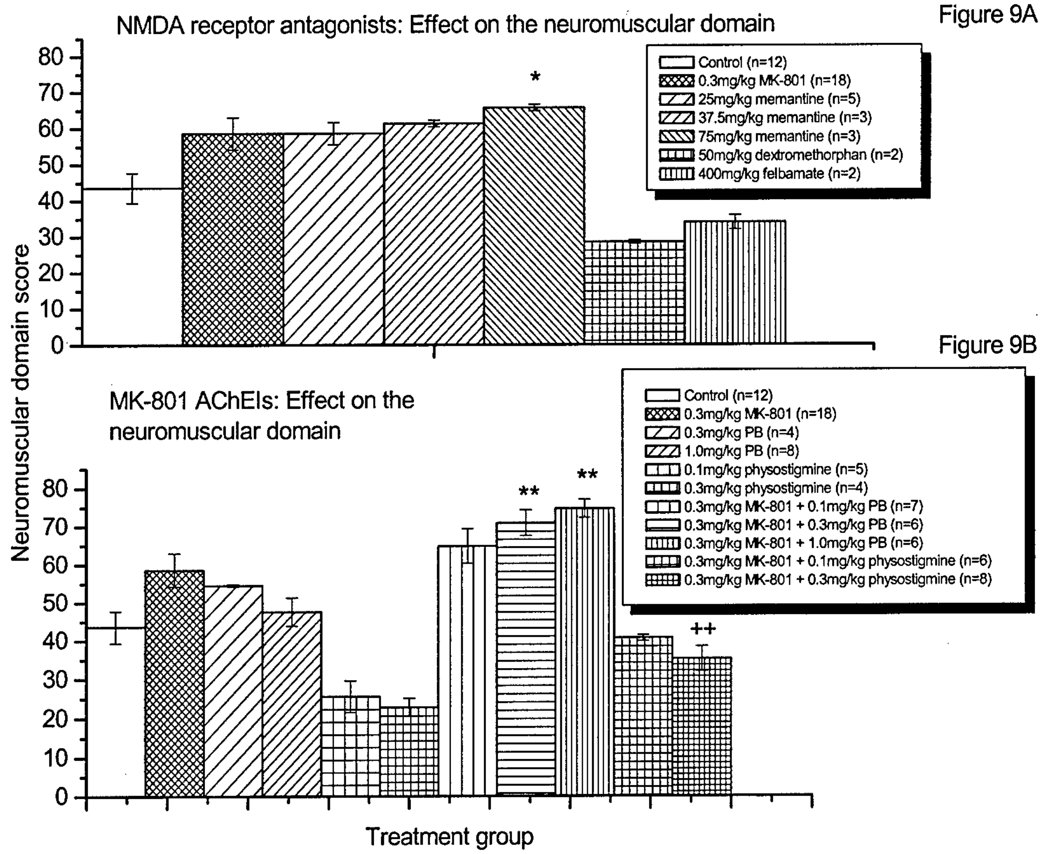


Figure 10

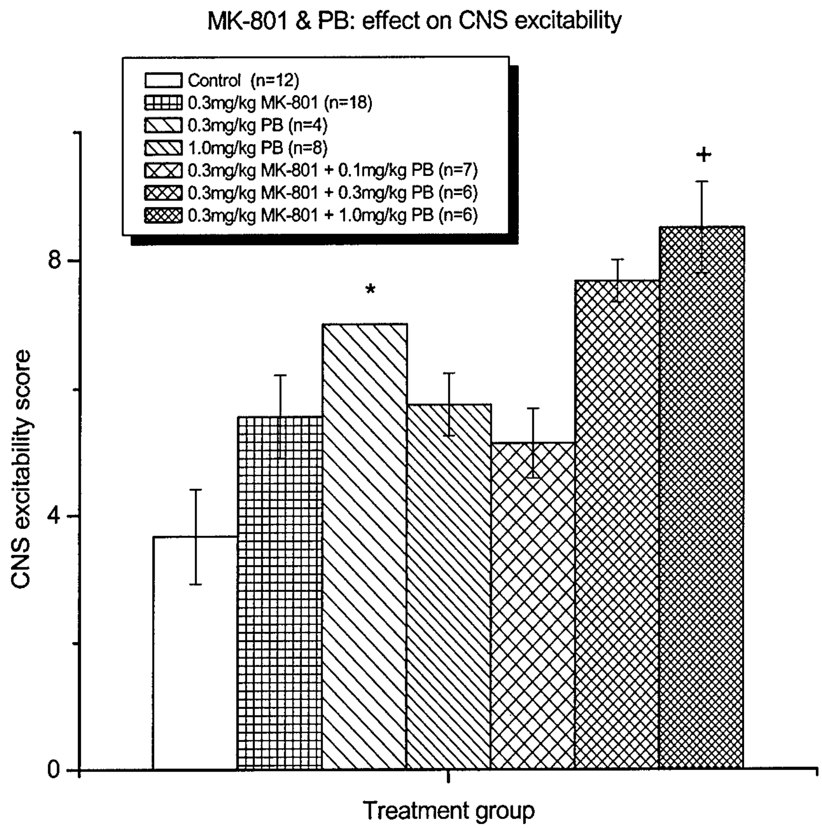
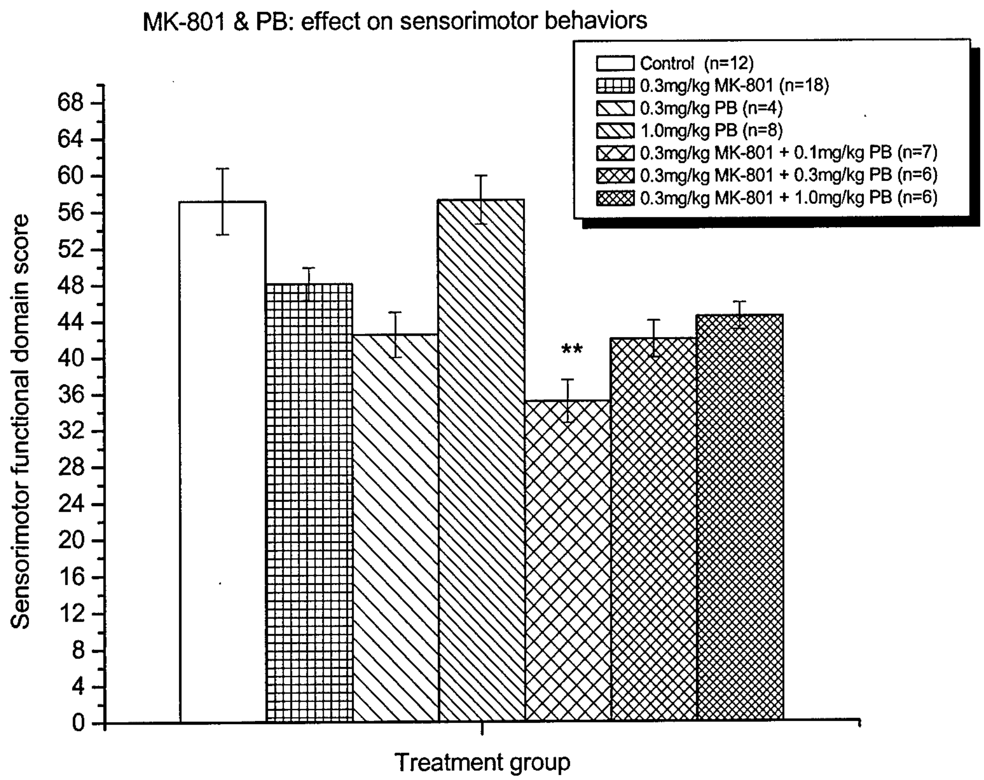

MK-801 & PB: effect on CNS excitability
Control (n=12)
0.3mg/kg MK-801 (n=18)
0.3mg/kg PB (n=4)
1.0mg/kg PB (n=8)
0.3mg/kg MK-801 + 0.1mg/kg PB (n=7)
0.3mg/kg MK-801 + 0.3mg/kg PB (n=6)
0.3mg/kg MK-801 + 1.0mg/kg PB (n=6)
CNS excitability score
0
4
8
*
+
Treatment group

Figure 11


MK-801 & PB: effect on sensorimotor behaviors
Control (n=12)
0.3mg/kg MK-801 (n=18)
0.3mg/kg PB (n=4)
1.0mg/kg PB (n=8)
0.3mg/kg MK-801 + 0.1mg/kg PB (n=7)
0.3mg/kg MK-801 + 0.3mg/kg PB (n=6)
0.3mg/kg MK-801 + 1.0mg/kg PB (n=6)
Sensorimotor functional domain score
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
**
Treatment group

II: <u>Histopathology:</u>

Memantine neurotoxicity and lethal interactions with the AChEI: pyridostigmine bromide.

Katherine H Jones, Suzanne Clark, Shirley X Guo-Ross, Elena Kuhn, and Wilkie A Wilson

(note: Part of this manuscript is being prepared for publication in the *Journal of Physiological and Experimental Therapeutics*)

Introduction

The neurotransmitter glutamate produces excitotoxicity than may cause neurodegeneration in acute brain injuries (head trauma, stroke, and epilepsy), and chronic neurodegenerative diseases (Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, and Parkinson's disease). This toxicity may be mediated by *N*-methyl-D-aspartate (NMDA) receptor activation. Accordingly, antagonists of NMDA receptors and channels (NMDA antagonist) were tested for neuroprotective efficacy, and both competitive (CPP) and non-competitive (MK-801, ketamine, phencyclidine (PCP), dextromethorphan, felbamate, and memantine) NMDA receptor antagonists protected against glutamate-induced neurotoxicity in acute brain injury models (Clifford et al., 1990, Chen et al., 1992, Wasterlain et al., 1992, Britton et al., 1997, Ikonomidou et al., 2000, Rao et al., 2001). Although NMDA receptor antagonists were neuroprotective under certain conditions (e.g., when pre-administered), their other effects proved to be unexpectedly problematic. Specifically, their action produces a state of receptor hypofunction, which in itself can induce neurodegeneration in vulnerable neurons (Olney et al., 1995).

Both competitive and noncompetitive NMDA receptor antagonists can induce receptor hypofunction in corticolimbic neurons, and can produce signs of neurotoxicity, including neuronal vacuolization, heat shock protein (HSP) expression and increased glucose metabolism (Kurumaji et al., 1989, Olney et al., 1991, Sharp et al., 1991). The extent of observed neuropathologies depends upon the degree of NMDA receptor hypofunction. Damage may be reversible with low affinity antagonists (or low doses of higher affinity antagonists) (Allen and Iverson, 1990). However, high or repeated doses of many NMDA receptor antagonists produce

signs of permanent damage, and also extend the pattern of neurodegeneration beyond the PC/RSC (Horvath and Busaki, 1993).

Mechanisms underlying NMDA receptor antagonist-induced neurotoxicity appear complex. Many agents attenuate NMDA antagonist-induced neurotoxicity, including anticholinergic, α2-noradrengergic and GABAergic agents (Olney et al., 1991, Kim et al., 1999). In contrast, few drugs exacerbated this toxicity; one of the strongest was pilocarpine, a cholinergic agonist (Corso et al., 1997).

The above experiments provided evidence for polysynaptic mechanisms of toxicity, by disinhibition of excitatory glutamatergic and cholinergic pathways that converge onto pyramidal neurons in the PC/RSC (Farber et al., 2002). In this model, tonic GABAergic inhibition modulates excitatory drive onto pyramidal cells from cholinergic muscarinic and glutamatergic AMPA receptors. These GABAergic neurons are, themselves, driven by tonic NMDA receptor activation. Thus, NMDA antagonists would block their excitatory input, reducing tonic inhibitory output.

These GABAergic neurons may be part of the local circuit or may include GABAergic interneurons in the basal forebrain and anterior thalamus (Farber et al, 2002). In addition, the circuit may include NMDA receptors on noradrenergic neurons that project from the locus coeruleus onto cholinergic neurons in the basal forebrain. If NMDA antagonists block GABA and noradrenaline release, then both excitatory cholinergic and glutamatergic pathways may become disinhibited, leading to excessive release of acetylcholine and glutamate onto PC/RSC pyramidal neurons. If disinhibition is sufficient, excitotoxicity occurs.

Given the importance of cholinergic exacerbation of this toxicity, we investigated the interactions of the acetylcholinesterase inhibitors (AChEI) PB and physostigmine with different NMDA receptor antagonists. AChEI's were of particular interest because they, as opposed to direct cholinergic agonists, are encountered in a wide range of clinical settings.

We chose a range of NMDA antagonists to investigate: MK-801, dextromethorphan, felbamate and memantine. MK-801 is the prototypical experimental NMDA receptor channel blocker. The neurotoxic effects of MK-801 are mediated by the dose, the age, and sex of the animal under investigation (Fix et al., 1993, Auer, 1996). The other agents we chose because of wide current (or future) clinical use. Dextromethorphan is an antitussive agent, used in cough medications; it is a low affinity, voltage-dependent NMDA receptor ion channel blocker

(reviewed by Britton et al., 1997). Felbamate is a broad-spectrum anticonvulsant agent- rarely used for the treatment of seizures. Felbamate is a voltage-independent noncompetitive NMDA receptor antagonist; however it's site of action on the receptor is a matter of dispute. Memantine is used in Europe to treat dementias, neuropathies, and Parkinson's disease; it is a low affinity voltage-dependent NMDA receptor ion channel blocker (reviewed by Parsons et al., 1993).

The AChEI agent, PB was chosen initially because of its wide use in the Persian Gulf War, and as an example of an agent not expected to cross the blood brain-barrier due to a positively charged ammonium group (reviewed by Chaney et al., 1999). Thus, we did not expect to see effects in the CNS of this drug. However, some reports describe PB-induced CNS effects, including changes in gene expression (Friedman et al., 1996) and neuronal apoptosis (Li et al., 2000). PB reversibly inhibits 30-40% of the AChE in the peripheral nervous system (Blick et al., 1991), and was therefore given to military personnel during the Gulf War conflict as a prophylactic treatment against organophosphate poisoning (Persian Gulf Veterans Coordinating Board, 1995); it has also been successfully used to treat myasthenia gravis for many years. We also chose to investigate the effects that physostigmine may have in potentiating NMDA receptor-mediated neurodegeneration, specifically because this AChEI agent does cross the blood brain barrier to act in the CNS.

This report describes the use of the neurodegeneration marker, Fluoro-Jade B (FJ-B) to investigate the histopathological effects of combining PB or physostigmine with different NMDA receptor antagonists. FJ-B is a fluorochrome that produces results comparable to silver staining- to specifically detect degenerating neurons (Schmued et al., 2000).

Methods

The drugs used in this study were from Sigma-Aldrich, St. Louis, MO: pyridostigmine bromide (PB): 3-(Dimethylaminocarbonyloxy)-1-methylpyridinium bromide, physostigmine hemisulfate (eserine hemisulfate), (+)-MK-801 hydrogen maleate: (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine), dextromethorphan hydrobromide monohydrate: (d-3-Methoxy-N-methylmorphinan); felbamate: (2-phenyl-1,3-propanediol dicarbamate), and memantine: (3,5-dimethyladamantane). Paraformaldehyde, potassium permanganate, sodium chloride, dibasic sodium phosphate and monobasic sodium phosphate,

were also purchased from Sigma-Aldrich, St. Louis, MO. Fluorojade-B was purchased from Histo-Chem Inc., Jefferson, AR.

Results from previous studies show that female rats are more sensitive than male rats to NMDA receptor induced neuronal toxicity (Olney et al., 1989, Auer, 1996), we therefore only used adult female Sprague-Dawley retired breeder rats for this study. Retired breeder rats are typically 3-6 months old. Rats were ordered from Charles River (Raleigh, NC) and housed in groups of two-three animals in clear polycarbonate cages (27.9 X 27.9X 17.8 cm^3) on a 12 hr light/dark cycle at the Durham Veterans Affairs Medical Center (VAMC) Vivarium. All animal procedures were approved by the Durham VAMC Institutional Animal Care and Use Protocol. The VAMC is an AAALAC accredited institution. Three days after arrival, the animals were individually weighed and randomly assigned to control or treatment groups. The average rat weight was 382.0g (+/- 8.8, SEM). Rats were given drugs or vehicle using intra-peritoneal (i.p.) or subcutaneous (s.c.) injections in a volume of 1.0ml/kg (in some cases, to ensure the drug was properly dissolved, drugs were given in a larger volume- eg. 2.0ml/kg was given to animals that received 50mg/kg dextromethorphan). Drugs were prepared fresh on injection day; PB, physostigmine, MK-801, dextromethorphan and memantine were each dissolved in sterile 0.9% saline. Felbamate was mixed in DMSO to 400 or 300mg/ml, was heated and stirred until it dissolved, and was finally injected at 1ml/kg. In each experiment, rats were first injected with the NMDA receptor antagonist of interest (MK-801, dextromethorphan, felbamate or memantine), followed within 15 minutes by injection with saline, PB, or physostigmine. We used the current literature to choose dose ranges for each drug; for the NMDA receptor antagonists, we based our doses on those used in rat neuroprotection studies. The low PB dose we used was roughly equivalent to that used clinically in humans to inhibit approximately 20% of peripheral cholinesterase (Servatius et al., 1998). The doses given for each drug were as follows: PB: 0.1, 0.3 and 1.0mg/kg, physostigmine: 0.1 and 0.3mg/kg, 1.0mg/kg, (+)MK-801: 0.3mg/kg, dextromethorphan: 10, 20 and 50mg/kg, felbamate: 100, 300 and 400mg/kg, and memantine: 25, 37.5, 50 and 75mg/kg. Control animals were given 0.9% saline (s.c.) followed by a second 0.9% saline injection (i.p.).

Three days after drug injections the rats were weighed, deeply anesthetized by Halothane® (J.A. Webster, Inc., Sterling, MA) inhalation, and transcardially perfused with 150-200ml of 0.9% saline with 0.1% heparin (heparin sodium injection; Elkins-Sinn, Cherry Hill,

NJ), followed by 350-400ml 4% buffered paraformaldehyde. The brains were removed and post-fixed in 4% buffered paraformaldehyde overnight, then transferred to 0.1M phosphate buffer (PB) for 24 hours; finally, the brains were soaked in 30% sucrose in 0.1M PB until they sank. Each brain was cut into 40-micron thick sections using a cryostat in a rostral-to-caudel direction. Twelve serial sections (approximately every sixth section beginning at -0.3mm from bregma (Paxinos and Watson, 1986) were mounted directly to 1.0% gelatin-coated slides (300 Bloom gelatin- type A; Electron Microscopy Sciences, Fort Washington, PA). The remaining sections were transferred to 0.1M PB and stored at 4°C for future use. The sections were stained with the fluorochrome, Fluoro-Jade B (FJ-B) using methods previously described (Schmued and Hopkins, 2000). Briefly, the sections mounted from the cryostat were allowed to dry on slides overnight at room temperature, then were sequentially processed in the following solutions: 100% ethanol (3 min), 70% ethanol (1 min), deionized water (1 min), 0.06% potassium permanganate (7 min, slow shaking), deionized water (1 min), 0.005mg/ml FJ-B (in 0.1% acetic acid) (17 min slow shaking) in the dark, then deionized water (three times, one minute each). Slides were then sequentially dehydrated in 70%, 90% and 100% ethanol (5 min each solution, two times in 100% ethanol). Slides were finally placed in xylene (5 min, two times), and cover-slipped with distyrene plasticizer xylene (Electron Microscopy Sciences, Fort Washington, PA). The stained sections were examined with a Zeiss Axiophot microscope using epifluorescence and a FITC filter. Neurotoxicity was assessed by taking the sum of positively stained neurons in the posterior cingulate cortex- retrosplenial cortex (PC/RSC) region across 12 serial sections.

Statistical analysis

Origin was used to calculate means and standard error of the means (SEM) and to present graphic representation of the data. Histological data is presented as means (+/- SEM). All raw data (the total number of FJ-B positive cells across twelve serial sections) was averaged, and the standard error of the means determined for each treatment group. An additional one-way ANOVA with a Tukey HSD post-hoc test was done on the histological data using SPSS.

Results

As previously reported (Clark et al., 2000), the AChEIs: PB and physostigmine were tested in combination with the NMDA receptor antagonist MK-801 to determine whether these

prototypical AChEIs potentiate the neurotoxicity exhibited by MK-801 alone. Reports by Olney (1991), and Corso et al., (1997) show that the neurotoxic effects of NMDA receptor antagonists are mediated by the muscarinic cholinergic system. Specifically, the muscarinic cholinergic antagonists atropine and scopolamine blocked vacuolization and HSP-70 protein induction by MK-801 in the rat retrosplenial cortex (PC/RSC) (Olney 1991). Also, the addition of the non-selective muscarinic cholinergic agonist pilocarpine exacerbated the degree of cell death in the brains of PCP-exposed rats (Corso et al., 1997). The AChEIs of interest in this study differ from one another in that PB is not expected to cross the blood-brain barrier, whereas physostigmine has access to the CNS. Physostigmine was used in these studies in combination with MK-801 to compare results with those of PB-exposed animals.

Each agent used in this study was first tested individually in adult female rats to determine whether they induce neurotoxicity in the PC/RSC- as evidenced by positive FJ-B staining of necrotic neurons in the PC/RSC. Exposure to either saline (n=7), 1.0mg/kg PB (n=8), or 0.3mg/kg physostigmine (n=3), did not generate any FJ-B positive cells (data not shown). With the exception of MK-801 (used only at one dose- 0.3mg/kg), we tested different doses of each NMDA receptor antagonist to determine whether they induce FJ-B positive staining (Fig 12). As expected, the low dose (0.3mg/kg) of MK-801 induced neurotoxicity in the PC/RSC (12A); although high doses of dextromethorphan (12B) and felbamate (12C) did not induce a neurotoxic response, a high dose (75mg/kg) of memantine did induce neurodegeneration (12D). Based on these results, we tested several lower doses of memantine and determined that all doses of memantine tested induced neurodegeneration in the PC/RSC in all exposed animals (Fig. 13). As shown, the mean number of FJ-B positive cells increased as the memantine dose increased: animals given a 25mg/kg dose had an average of 39.33 FJ-B positive cells/12 sections (+/- 15.98 SEM, n=12), animals given a 37.5mg/kg dose had an average of 147.0 FJ-B positive cells/12 sections (+/- 46.65 SEM, n=6), animals given a 50mg/kg dose had an average of 300.83 FJ-B positive cells/12 sections (+/- 71.58 SEM, n=6), and animals given a 75mg/kg dose had an average of 772.83 FJ-B positive cells/12 sections (+/- 311.52 SEM, n=6).

Tomitaka et al., (1996), showed that memantine induced heat shock protein both in the PC/RSC and in the dentate gyrus- based on these results, we examined the dentate gyrus in our high dose animals (75mg/kg), but did not note the presence of FJ-B positive cells.

In the remaining experiments, we sequentially tested each NMDA receptor antagonist with and without co-exposure to PB or to physostigmine. Several animals died in this series of experiments (Table 3); of the three animals that received both PB and 50mg/kg dextromethorphan, two died within the first hour after injection. Also, co-exposure of memantine and PB proved lethal for many animals within the first hour after injection: a 75mg/kg dose of memantine co-injected with PB caused three of six animals to die, a 50mg/kg memantine dose caused one out of three animals to die, a 37.5mg/kg dose caused four out of five animals to die, and a 25mg/kg memantine dose caused one out of twelve animals to die. None of the 31 animals that received memantine alone at any dose died. Also, none of the animals that received MK-801 alone (12 animals) or MK-801 in combination with PB (6 animals) died.

To improve animal survivability, we continued the remaining experiments using the lowest tested dose of memantine (25mg/kg) in animals co-exposed to PB or physostigmine. The low dose of MK-801 used in these experiments was based on previous (unpublished) results from this lab, which show that 0.3mg/kg of MK-801 reliably induces FJ-B positive neurons in the PC/RSC region in the adult female rat brain. Also, we wanted to have minimal but statistically significant neuronal death to detect any modulating effects that PB might have on MK-801-induced neurotoxicity. Histological data summarized from animals exposed to MK-801 alone or co-exposed to 0.3mg/kg MK-801 and 1.0mg/kg PB or to 0.1 and 0.3mg/kg physostigmine is presented in figure 14A. Exposure to 0.3mg/kg MK-801 alone induced an average of 268.1 FJ-B positive cells/12sections (+/- 107.02 SEM, n=12) in the PC/RSC. When combined with MK-801, PB confers some protection against MK-801 induced toxicity; animals that received MK-801 and PB had an average of 93.33 FJ-B positive cells/12 sections (+/- 23.64 SEM, n=6). A 0.1mg/kg dose of physostigmine has no effect on MK-801 mediated neurotoxicity; animals averaged 262.67 FJ-B positive cells/12 sections (+/- 44.65 SEM, n=6), however, by increasing the dose to 0.3mg/kg, the inclusion of physostigmine in co-exposed animals appears to exacerbate MK-801-mediated neurotoxicity in the PC/RSC; animals averaged 448.25 FJ-B positive cells/12 sections (+/- 257.39, n=4). PB also appears to also be neuroprotective in 25mg/kg memantine co-exposed animals; animals exposed to 25mg/kg memantine alone had an average of 39.33 FJ-B positive cells/12 sections (+/-15.98, n=12), while animals co-exposed to 25mg/kg memantine and 1.0mg/kg PB averaged 22.73 FJ-B positive cells/12 sections (+/-8.32, n=11) (Fig. 14B). 0.3mg/kg physostigmine appeared to ameliorate

25mg/kg memantine-induced neurotoxicity in co-exposed animals; the average number of FJ-B positive cells in co-exposed animals was 14.36 cells/12 sections (+/- 4.39 SEM, n=11).

In order to try to elucidate whether age may affect the degree of neurotoxicity induced by MK-801 or memantine, we made the assumption that an older animal weighs more than a younger animal. Thus, we plotted each animal's weight against it's histological score; determined by adding together the total number of FJ-B positive cells from 12 serial sections approximately 200um apart (figure 15). We did not know the exact age of each animal, instead, by ordering retired breeder rats, we received animals that ranged in age from 3-6 months. Younger animals that were not 'good' breeders were included with older animals ready to 'retire' form breeding. As shown, there was no clear correlation between the animal's weight and her expressed histopathology.

Discussion

The present study was designed to investigate the neurotoxic risks that are associated with the combined use of acetylcholinesterase inhibitors (AChEIs) and N-methyl-D-aspartate (NMDA) receptor antagonists. This is a particularly relevant topic- in that many people encounter AChEIs and NMDA antagonists in a wide range of settings. AChEI's exist as therapeutic drugs, insecticides, and as prophylactic agents against nerve gas poisoning. Drugs that posses NMDA antagonist activity include currently available therapeutic drugs (e.g. certain anticonvulsants and antitussives) and also as investigational drugs (e.g., drugs for Parkinson's disease, spasticity, chronic pain, dementias, and anticonvulsants effective against severe nerve gas-induced seizures). NMDA antagonist activity is also present in several drugs of abuse, including ethanol or PCP (angel dust). It is increasingly likely that agents from both of these groups will be co-administered, with potentially dangerous consequences, especially in life-threatening situations such as exposure to- or treatment for- nerve gas poisoning.

We chose to use PB and physostigmine in our preliminary experiments to test whether co-exposure of these AChEIs with the NMDA receptor antagonist MK-801 resulted in increased excitotoxicity. Our later studies specifically investigated the effects that PB and physostigmine may have in inducing neurotoxicity in PC/RSC neurons after they are co-exposed to various NMDA receptor antagonists that may be clinically or militarily encountered. Physostigmine was used as a representative AChEI that has activity in the CNS, as opposed to PB, which is

primarily active in the peripheral nervous system (PNS). PB is a topically relevant AChEI, as it may be used as a prophylactic agent against nerve agents in future wars. Unlike physostigmine, PB is not expected to cross the blood-brain barrier (Birtley et al., 1966). However, a study by Friedman et al., (1996) suggests that under conditions of stress (forced swimming stress in mice) PB may cross the blood-brain barrier to inhibit AChE in the brain.

We found that PB modulates the effects of NMDA receptor antagonists in two ways: First, several animals co-exposed to a modest dose (1.0mg/kg) of PB and the NMDA receptor antagonists: dextromethorphan or memantine died. Second, the surviving PB-memantine co-exposed animals had fewer FJ-B positive cells than those exposed to memantine alone. This second result was similar to that seen in PB-MK-801 co-exposed animals, and suggests that PB is modestly protective against NMDA receptor-mediated neurotoxicity.

In addition to PB mediated effects, we also found that co-exposure of animals to physostigmine exacerbated pyramidal neuron neurodegeneration in the PC/RSC in animals exposed to the NMDA receptor antagonist MK-801. Our initial studies determined that a low dose of MK-801 (0.3mg/kg) consistently produced FJ-B positive cells in the PC/RSC, although there was quite a bit of variability between animals in the numbers of positive cells. We then determined that (0.3mg/kg) physostigmine increased the number of positive FJ-B cells in animals co-exposed to MK-801. Thus, as expected, NMDA receptor-blockade by MK-801, combined with increased ACh concentrations (by physostigmine co-exposure) produced on average, more FJ-B positive cells than did MK-801 exposure alone. However, we found a contradictory result in 25mg/kg memantine-0.3mg.kg physostigmine co-exposed animals-physostigmine appeared to ameliorate memantine-induced neurotoxicity. It is possible that a higher dose of physostigmine would induce a neurotoxic response similar to that seen in 0.3mg/kg MK-801-0.3mg/kg physostigmine co-exposed animals; a 25mg/kg dose of memantine is clearly not equivalent to a 0.3mg/kg dose of MK-801. It is possible that these doses of memantine and physostigmine do not place vulnerable pyramidal neurons near the threshold required to induce excitotoxicity.

We then tested whether PB produced a similar degree of neurotoxicity in animals co-exposed to MK-801 as we found that when we combined physostigmine with a low dose of MK-801. We found that PB may be somewhat neuroprotective, as fewer FJ-B cells were evident on average in the co-exposed animals. Thus, PB appears to ameliorate the excitotoxic effect of

MK-801 in mature female rats. We then tested a series of additional NMDA receptor antagonists to establish whether they may induce excitotoxicity in the PC/RSC, and additionally, whether co-exposure to PB may ameliorate or exacerbate a toxic response. We chose to investigate the NMDA receptor antagonists: dextromethorphan, felbamate, and memantine. All of these therapeutic drugs have been described as neuroprotective compounds in either rat focal ischemia models (Wasterlain et al., 1992; Britton et al., 1997), or induced traumatic brain injury in the rat (Rao 2001); both are conditions that induce glutamate-mediated injury to cortical cells surrounding the experimental insult.

Dextromethorphan is an antitussive non-competitive NMDA receptor antagonist. However, it is not currently known whether neuroprotection by dextromethorphan against glutamate toxicity is primarily due to a direct interaction with the NMDA receptor (Britton et al., 1997). Our results did not suggest that dextromethorphan initiated an excitotoxic response by PC/RSC cells. We also did not detect any interaction between dextromethorphan and PB in co-exposed animals.

Felbamate is used as an anticonvulsant agent for epilepsy. Felbamate has the properties of a non-competitive NMDA receptor antagonist, but its exact site of action on the NMDA receptor is a matter of dispute. It has been proposed to bind within the ion channel (Rho et al., 1994), at the glycine site (Koek & Colpaert 1990), or none of the above (White et al., 1992). Our histological results did not indicate that felbamate had any excitotoxic effects on cells in the PC/RSC; nor did we detect any interaction between felbamate and PB.

Memantine is used clinically in United States for Parkinson's disease (reviewed by Kornhuber and Weller 1996), and is in phase three trials for clinical use against vascular dementias (Orgogozo 2002). It is a non-competitive antagonist that binds within the NMDA receptor ion channel (Kornhuber et al., 1989; Bormann 1989; Parsons et al., 1993). Memantine induces heat-shock protein in the PC/RSC and also in the dentate gyrus (Tomitaka et al., 1996); a result similar to others in which non-competitive NMDA receptor antagonists induced heat shock proteins in the PC/RSC, for example PCP (Corso et al., 1997), MK-801 (Berger et al., 1994), dextrorphan, (Lan et al., 1997), and ketamine (Sharp et al., 1991). Thus, we were not surprised to find that memantine exposure produced an excitotoxic response, as demonstrated by the presence of FJ-B positive cells in the PC/RSC.

We did not predict that low-dose PB would prove lethal in dextromethorphan and memantine co-exposed animals. We were surprised that all doses of memantine caused death, since on average, 0.3mg/kg of MK-801 produced as many FJ-B positive cells in the PC/RSC as did 50mg/kg memantine, yet MK-801-PB co-exposed animals all survived. Thus, the co-exposed animals deaths appear to be due to mechanisms other than PC/RSC excitotoxicity. In each case, the dying animal appeared to experience seizures minutes after the PB injection, and died several minutes later. To determine that death was due to seizures, future studies should monitor EEG activity in co-exposed animals.

It is important to note that although most of the memantine doses used in our experiments were atypically high, due to differences in metabolism between rats and humans, the acute moderate dose we used (25mg/kg) is only modestly higher than the equivalent dose in the human (reviewed by Chen et al., 1998). Our results also indicate that the 25mg/kg dose in the adult female rat is near a threshold of excitotoxicity. Perhaps due to natural variations between animals, some animals appeared to be more sensitive than others to memantine.

Our results regarding the excitotoxic nature memantine contradict the apparent safety and efficacy of this drug. As reviewed by Parsons et al., (1999), memantine has been well tolerated and in use clinically for over 15 years. Memantine is an open channel blocker (Chen et al., 1992), therefore it only blocks the NMDA receptor after the cell depolarizes; and, due to its low affinity, the next depolarization allows memantine to leave the ion channel. It is thought that the rapid blocking and unblocking action of memantine inhibits the overall activity of NMDA receptors, leaving enough receptors unblocked at any one time to facilitate cognitive function, and to suppress cholinergic function enough to avoid a neuronal excitotoxic response. Chen et al., (1998) hypothesized that memantine is most effective during pathological conditions that result in increased extracellular glutamate concentrations- to suppress the resulting cholinergic hyperactivation of neurons.

That PB reduces the neurodegenerative effects induced by two of the NMDA receptor channel blockers tested was an unexpected result, we predicted that MK-801 and memantine would increase ACh in the CNS (mediated by NMDA receptor blockade), and the addition of the AChEI PB, should further increase ACh concentrations to induce hyperactivity in cholinergic neurons. Our results suggest that PB acts upon the CNS, perhaps to re-establish disinhibited tonic inhibition pathways in PC/RSC neurons. GABAergic agents are considered neuroprotective

against NMDA receptor-mediated excitotoxicity (Olney et al., 1991, Nakao et al., 2003). Results from a recent study by Santos et al., (2003) suggest that PB facilitates GABAergic transmission directly, by binding to muscarinic receptors on GABAergic neurons. It is possible that PB modulates CNS activity *via* the PNS through other mechanisms. Others have reported PB-mediated CNS effects: Chaney et al., (1999) described experiments in which PB-induced seizures in mice could not be suppressed by centrally acting anticonvulsants, yet were inhibited by the muscarinic antagonist, atropine; Li et al., (2000) also determined that PB-mediated apoptosis in the rat is inhibited by pre-exposure of the animals to atropine.

Vulnerability to memantine in the rat may be a function of age, sex or genetic variability. For example, Farber et al., (1995) suggested that increased NMDA receptor hypofunction in the rat is an age-related result, therefore it is possible that remaining functional NMDA receptors in an older animal may be inhibited by a standard dose of memantine. Also, Serra, et al, (1994) determined that although there are fewer NMDA receptors in the aged rat, both glutamate and glycine increased the ability of the NMDA receptor to bind MK-801, this suggests that pathological conditions that increase extracellular glutamate levels may also increase memantine's affinity to bind the NMDA receptor, leaving too few NMDA receptors unblocked, leading to an excitotoxic reaction.

Sex probably also plays a role in increased vulnerability to NMDA receptor antagonists (Olney et al., 1989, Auer, 1996). Smith, (1989) determined that estrodiol modulates NMDA receptor binding by potentiating binding of the receptor to the agonist, NMDA. This suggests another possible mechanism for neuronal overexcitation- if NMDA receptors are already slightly hyperactive (by estrogenic activity) and then are confronted with memantine, too many receptors may be blocked to facilitate cholinergic inhibition to cortical neurons.

There is current interest in the use of memantine to treat Alzheimer's disease (AD) in the United States. One hallmark of AD is a loss of cholinergic neurons. Thus, treatment includes increasing concentrations of ACh by inhibiting AChEs. In addition, AD has been proposed to involve over-activation of NMDA receptors (reviewed by Rogawski and Wenk, 2003). Consequently, new therapies are aimed at decreasing hyperactivation of NMDA receptors. By combining these compounds, the hope is to ameliorate the cognitive deficits of AD and slow disease progression by sparing cholinergic neurons from glutamate insult. However, Olney and colleagues recently reported that the combination of memantine and AChEIs negatively affect

animal learning (Creeley et al., 2003). The brain areas most affected by memantine in the rat are, in humans, involved in subtle behaviors and integrative processes- damage to these areas may not be obvious in an individual with advanced AD to whom such drugs may be given. Nor may these patients have sufficient verbal skills to report distressing symptoms. Caregivers may assume that drug-induced changes in neurological status are a progression of AD and may not suspect a neurotoxic drug interaction. Future, more extensive studies are urgently needed.

Figure legends:

Table 3: NMDA receptor antagonist-mediated lethality in animals co-exposed to pyridostigmine. Data are presented as the number of rats that survived injection with the NMDA receptor antagonists: MK-801, dextromethorphan, felbamate, or memantine, and co-injected with either saline or 1.0mg/kg pyridostigmine. The numerator of each value describes the number of surviving animals; the denominator describes the total number injected.

Figure 12. Effect of NMDA receptor antagonists on the PC/RSC region in the adult female rat brain. Representative Fluoro-Jade B (FJ-B) stained brain sections from rats given a single s.c. dose of : 0.3mg/kg MK-801 (12A), 50mg/kg dextromethorphan (12B), 400mg/kg felbamate (12C), or 37.5mg/kg memantine (12D). Rats were injected three days prior to perfusion-fixation. No FJ-B positive cells were evident in sections from dextromethorphan or felbamate treated rats (B&C). Positive stained FJ-B cells are easily scored in sections A&D. Photographs were taken using a 10X objective using a FTIC filter. The PC/RSC region is shown with the pial surface to the right of the photograph. The white bar in image 12A represents 32 micrometers.

Figure 13. Concentration-dependent effect of memantine in the adult female rat PC/RSC. Memantine-injected rats were injected three days prior to perfusion-fixation, and are compared to saline-injected control animals. Bar graph values represent the mean number of FJ-B positive cells, +/- SEM. Saline-injected rats never expressed FJ-B positive cells. Photographs B-E were taken in the PC/RSC region shown in drawing A. The average number of FJ-B positive cells increased as the rats were exposed to increasing memantine concentrations. Note the FJ-B positive processes projecting from the cell body. All images are shown with the pial surface

oriented to the right. Photographs were taken using a digital camera and a 25X water-immersible objective under a FTIC filter. The white bar in image 13B represents 14 micrometers

Figure 14. Effect of pyridostigmine on NMDA antagonist-mediated neurotoxicity in the adult female rat PC/RSC. A: Animals were given a single s.c. injection of 0.3mg/kg MK-801- followed by a single i.p. injection of 1.0mg/kg pyridostigmine or saline, or animals were given B: a single s.c. injection of 25mg/kg memantine- followed by a single i.p. injection of 1.0mg/kg pyridostigmine or saline. Injections were given three days prior to perfusion-fixation. Bars represent the mean number of positive FJ-B cells across 12 serial sections approximately 200um apart (+/- SEM). Note the difference in scale between graphs A&B along the Y-axis. Note that PB appears to ameliorate the effects of MK-801 in co-exposed animals.

Figure 15. Scatter plot. Weight *vs.* histopathology: Mk-801 and memantine. Animals were given either a single s.c. injection of: saline, 0.3mg/kg MK-801, 25 or 75mg/kg memantine, followed by a single i.p. injection of saline. Each animal's average weight (taken over four days) was plotted against that animals histopathology score (the mean number of positive FJ-B cells across 12 serial sections approximately 200um apart). Note that there does not appear to be a correlation between an animal's weight and her histopathology score.

Table 3

NMDA antagonist:	Co-injected with: Saline		Co-injected with: Pyridostigmine (1.0mg/kg)	
MK-801	Dose	Survivors	Dose	Survivors
	0.3mg/kg	12/12	0.3mg/kg	6/6
Dextromethorphan	Dose	Survivors	Dose	Survivors
	50mg/kg	2/2	50mg/kg	1/3
Felbamate	Dose	Survivors	Dose	Survivors
	400mg/kg	2/2	400mg/kg	2/2
Memantine	Dose	Survivors	Dose	Survivors
	25mg/kg	12/12	25mg/kg	11/12
	37.5mg/kg	7/7	37.5mg/kg	1/5
	50mg/kg	6/6	50mg/kg	2/3
	75mg/kg	6/6	75mg/kg	3/6

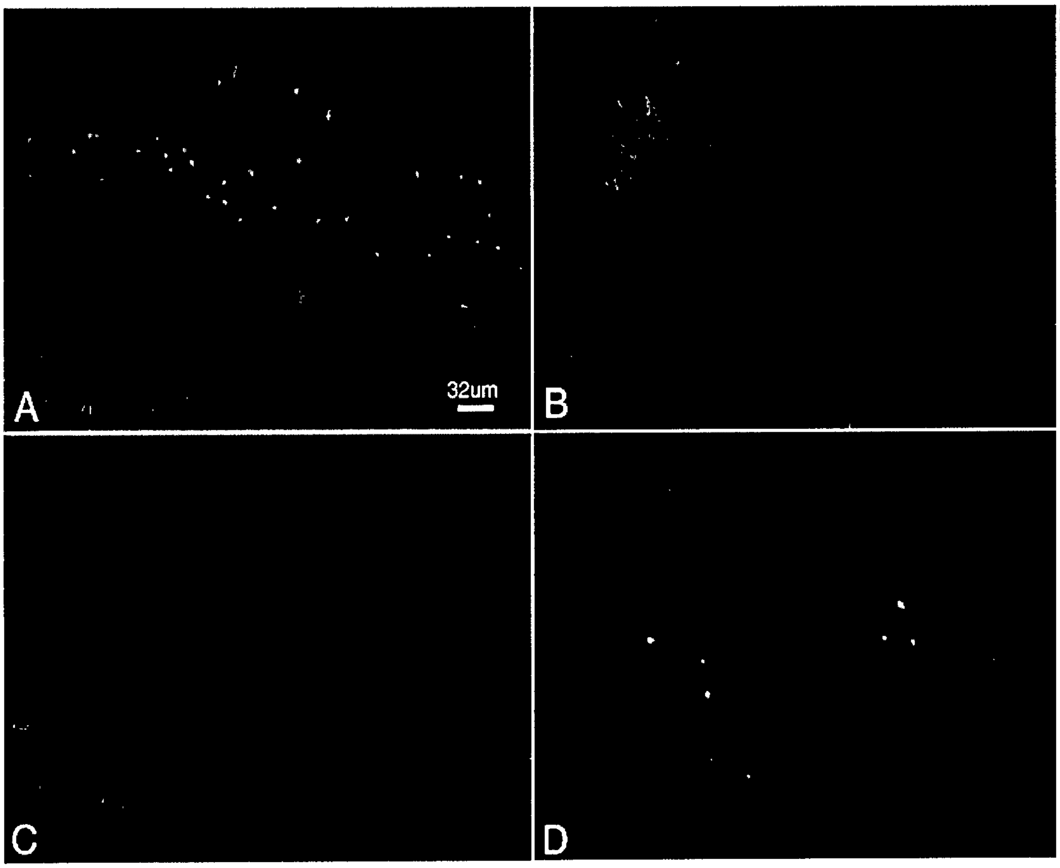
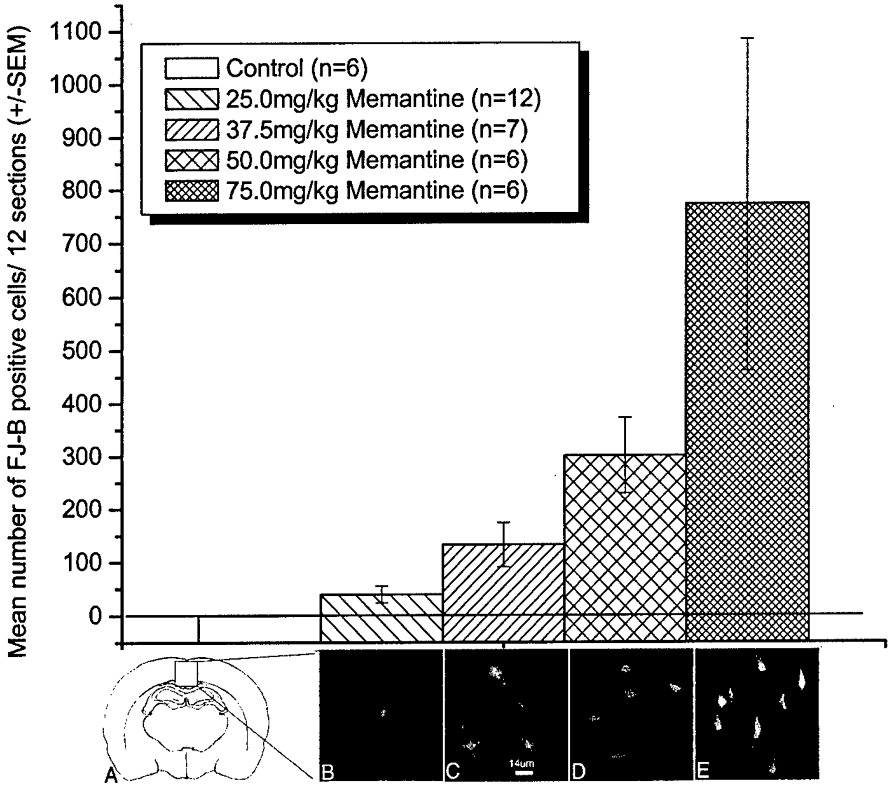


Figure 12

Figure 13



Mean number of FJ-B positive cells/ 12 sections (+/-SEM)
1100
1000
900
800
700
600
500
400
300
200
100
0
Control (n=6)
25.0mg/kg Memantine (n=12)
37.5mg/kg Memantine (n=7)
50.0mg/kg Memantine (n=6)
75.0mg/kg Memantine (n=6)
A
B
C
14um
D
E

Figure 14

The interaction of AChEIs: PB and physostigmine on MK-801 and memantine-mediated neurotoxicity

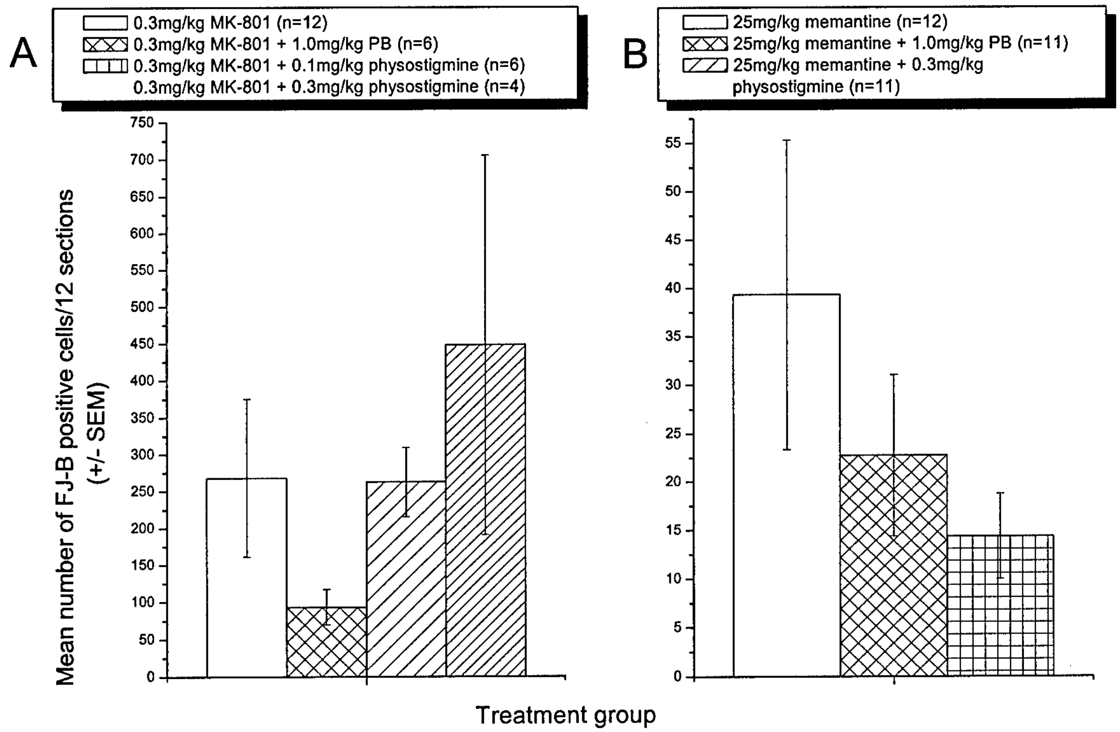
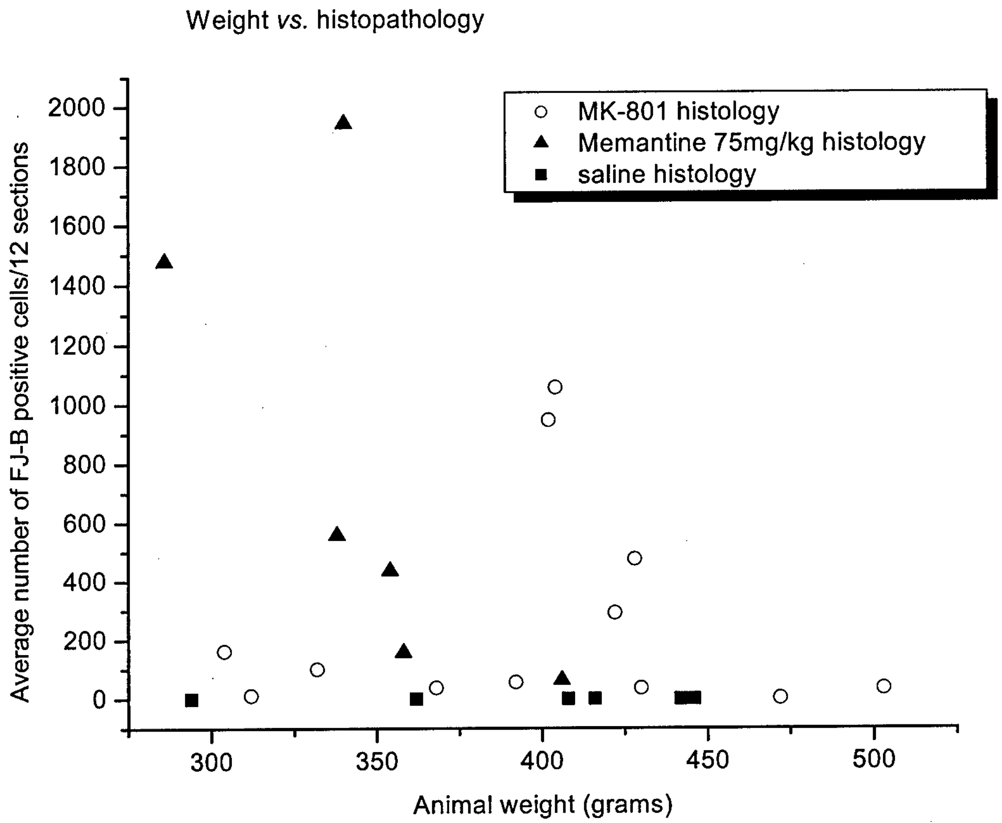


Figure 15


Weight vs. histopathology
MK-801 histology
Memantine 75mg/kg histology
saline histology
Average number of FJ-B positive cells/12 sections
2000
1800
1600
1400
1200
1000
800
600
400
200
0
300
350
400
450
500
Animal weight (grams)

III: <u>Histopathology and behavior:</u>

The neurotoxic consequences of exposure to the NMDA receptor antagonists MK-801 and memantine: A correlation between drug-induced behavioral changes determined by a functional observation battery and histopathology in the rat posterior cortex and retrosplenial cortex.

Katherine H Jones, Suzanne Clark, Elena Kuhn, and Wilkie A Wilson

Introduction

The effects of different neurotoxicants can be distinguished by observing the specific effects on behaviors, which can be measured using a functional observation battery (FOB). For example, non-competitive NMDA receptor antagonists induce specific behavioral changes that are not induced by competitive NMDA receptor antagonists (Bubser et al., 1992).

A complete neurotoxic screen includes an assessment of changes in specific behaviors, changes in motor behaviors, and finally, a histopathologic analysis of the agent of interest (Moser, 1990, EPA, 1998). Thus, we have investigated several neurobehavioral changes induced by the NMDA receptor antagonists: MK-801 and memantine, and compared them to the histopathologic changes for each of several animals treated with either MK-801 or memantine. This was done to try to correlate specific MK-801 and memantine-induced behaviors with the resulting histopathology of the PC/RSC brain region in the adult female rat.

Methods

The drugs used in this study were from Sigma-Aldrich, St. Louis, MO: (+)-MK-801 hydrogen maleate: (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine), and memantine: 3,5-dimethyladamantane. Paraformaldehyde, potassium permanganate, sodium chloride, dibasic sodium phosphate and monobasic sodium phosphate, were also purchased from Sigma-Aldrich, St. Louis, MO. Fluorojade-B was purchased from Histo-Chem Inc., Jefferson, AR.

Results from previous studies show that female rats are more sensitive than male rats to NMDA receptor induced neuronal toxicity (Olney et al., 1989, Auer, 1996), we therefore only used adult female Sprague-Dawley retired breeder rats for this study. Retired breeder rats are

typically 3-6 months old. Rats were ordered from Charles River (Raleigh, NC) and housed in groups of two-three animals in clear polycarbonate cages (27.9 X 27.9X 17.8 cm^3) on a 12 hr light/dark cycle at the Durham Veterans Affairs Medical Center (VAMC) Vivarium. All animal procedures were approved by the Durham VAMC Institutional Animal Care and Use Protocol. The VAMC is an AAALAC accredited institution. Three days after arrival, the animals were individually weighed and randomly assigned to control or treatment groups. The average rat weight was 382.0g (+/- 8.8, SEM). Rats were given drugs or vehicle using subcutaneous (s.c.) injections in a volume of 1.0ml/kg. Drugs were prepared fresh on injection day, MK-801 and memantine were each dissolved in sterile 0.9% saline. The doses given for each drug were, MK-801 0.3mg/kg, and memantine: 25 and 75mg/kg. Control animals were given 0.9% saline (s.c.). Animals were monitored for behavioral changes at 1 hour, 4 hours, 24 and 48 hours post-drug exposure, and then were sacrificed to process the brains for histopathologic analysis 72 hours post-drug exposure. The methods used for behavioral assessment of drug-exposed animals were as described above and the methods used for histopathological analysis were also as described above.

Results and Discussion

Scatter plots were made by choosing behaviors which seemed to be modified primarily by the NMDA receptor antagonists MK-801 and memantine (determined in earlier studies described above). These two agents produced FJ-B positive cells in the PC/RSC region of the adult female rat brain. Each animal's behavioral score in the positional passivity, neuromuscular, and bizarre behavior domains was plotted against her histopathology score.

We found that positional passivity, and to some extent, neuromuscular behaviors reflect the resulting histopathology seen in the PC/RSC brain region in rats exposed to a low dose of MK-801 (0.3mg/kg) and a high dose of memantine (75mg/kg). As shown in figure 16, it is clear that control animals and those exposed to a low dose of memantine (25mg/kg) are much less passive than those animals exposed to high dose memantine (75mg/kg) or to MK-801 (0.3mg/kg). The most passive animals had low passivity scores, and appeared to also express more FJ-B positive cells than the less passive animals. Similarly, as shown in figure 17, the animals that experienced the greatest deficit in neuromuscular domain behaviors were those that were exposed to MK-801 or to 75mg/kg memantine, although there is some overlap between the

two dose groups in the memantine treated animals. As shown, on average, those animals with a higher deficit tended to exhibit more FJ-B positive cells than less impaired animals. Figure 18 shows that bizarre behaviors exhibited by the animals do not reflect resulting neuropathology. The impairment in the positional passivity, and neuromuscular behaviors may describe increased ataxia, a common reaction induced by non-competitive NMDA receptor antagonists.

In general, the FOB results were not able to accurately predict resulting neuropathology in the PC/RSC brain region in our rats. This result is not too surprising, as the behaviors measured using the FOB may not reflect the behaviors changed in the PC/RSC brain region as a result of NMDA receptor antagonist-mediated excitotoxicity. The FOB was able to reflect non-competitive NMDA receptor antagonism, however. In addition, in some behaviors, a dose-response and agent specific effects were illuminated.

Figure legends:

Figure 16. Scatter plot. MK-801 and memantine: Comparison of positional passivity scores *vs.* histopathology. Changes in struggle behavior reflect the degree of NMDA receptor block in the PC/RSC. The raw histology score for each animal is compared to that animal's struggle behavior score. Raw histology scores are determined by counting the total number of FJ-B positive cells across 12 serial brain sections approximately 200um apart. Animals were given the FOB pre-test 24 hours prior to drug exposure, and then were given: a single s.c. injection of saline, 0.3mg/kg MK-801, or 25 or 75mg/kg memantine followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. Positional passivity scores were determined by adding together the scores determined at each post-injection time-point (see text and table 2). A decreased positional passivity score describes reduced struggle behavior as the animal is held by the tail 8-12 inches above the field surface. Note that the control and 25mg/kg memantine exposed animals were less passive on than animals that received 0.3mg/kg MK-801 or 75mg/kg memantine. Also note that in general, animals that are passive express more FJ-B positive cells than animals that struggle.

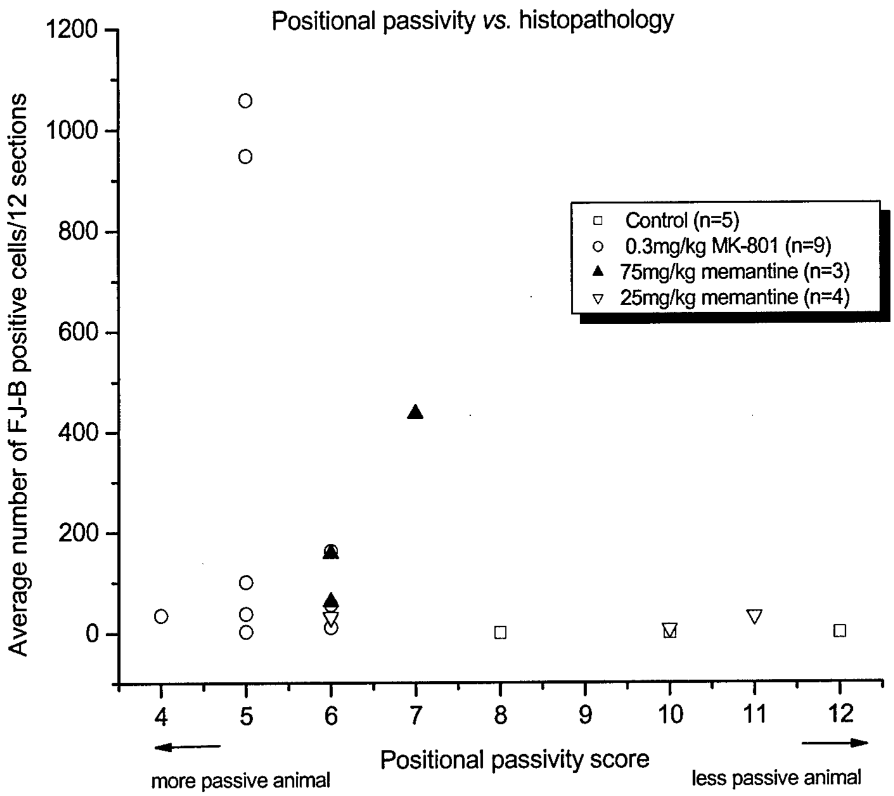
Figure 17. Scatter plot of MK-801 and memantine: Comparison of neuromuscular domain scores *vs.* histopathology. Behavioral changes in the neuromuscular domain do not appear to

reflect NMDA receptor-mediated neurotoxicity in the PC/RSC. The raw histology score for each animal was compared to that animal's behavioral score in the neuromuscular domain. Raw histology scores were determined by counting the total number of FJ-B positive cells across 12 serial brain sections approximately 200um apart. Animals were given the FOB pre-test 24 hours prior to drug exposure, and then were given: a single s.c. injection of saline, 0.3mg/kg MK-801, 25 or 75mg/kg memantine, followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. Neuromuscular domain scores were determined by adding together scores for each behavior within the neuromuscular domain (see text and table 2) using each post-injection time-point. The score for the neuromuscular domain is determined by assessing Straub tail, gait score, gait incapacity, limb rotation, grip strength, body tone, pencil grip, and extensor thrust. Note that an increased neurofunctional domain score did not reflect the degree of expressed neurotoxicity (the number of positive FJ-B cells), however, a low neuromuscular domain score (scores less than 60) did indicate the presence of few or no FJ-B positive cells. Also note that many animals given 25mg/kg or 75mg/kg memantine had their neuromuscular domain scores overlap, suggesting that there was no dose effect with this compound on these behavioral parameters. On average, 0.3mg/kg MK-801 induced a high degree of neurotoxicity in only 2/8 animals whose neuromuscular scores were above 70.

Figure 18: Scatter plot of MK-801 and memantine: Comparison of bizarre behavior scores *vs.* histopathology. The expression of bizarre behavior does not reflect resulting histopathology. The raw histology score for each animal is compared to that animal's bizarre behavior score. Raw histology scores are determined by counting the total number of FJ-B positive cells across 12 serial brain sections approximately 200um apart. Animals were given the functional observation battery (FOB) pre-test 24 hours prior to drug exposure, and then were given: a single s.c. injection of saline, 0.3mg/kg MK-801, or 25 or 75mg/kg memantine followed by a single i.p. injection of saline. Animals were further assessed using the FOB at 1 hour, 4 hours, 24 hours and 48 hours post-injection. Bizarre behavior scores were determined by adding together the scores determined at each post-injection time-point (see text and table 2). Note that all animals exposed to 0.3mg/kg MK-801 expressed bizarre behavior, and most memantine exposed animals did as well, however, there appears to be no correlation between the expression

of bizarre behavior and the expression of FJ-B positive cells in the PC/RSC, as some animals that did not appear to express bizarre behavior nevertheless expressed FJ-B positive cells in the PC/RSC.

Figure 16



Positional passivity vs. histopathology
Average number of FJ-B positive cells/12 sections
1200
1000
800
600
400
200
0
Control (n=5)
0.3mg/kg MK-801 (n=9)
75mg/kg memantine (n=3)
25mg/kg memantine (n=4)
4
5
6
7
8
9
10
11
12
more passive animal
Positional passivity score
less passive animal

Figure 17

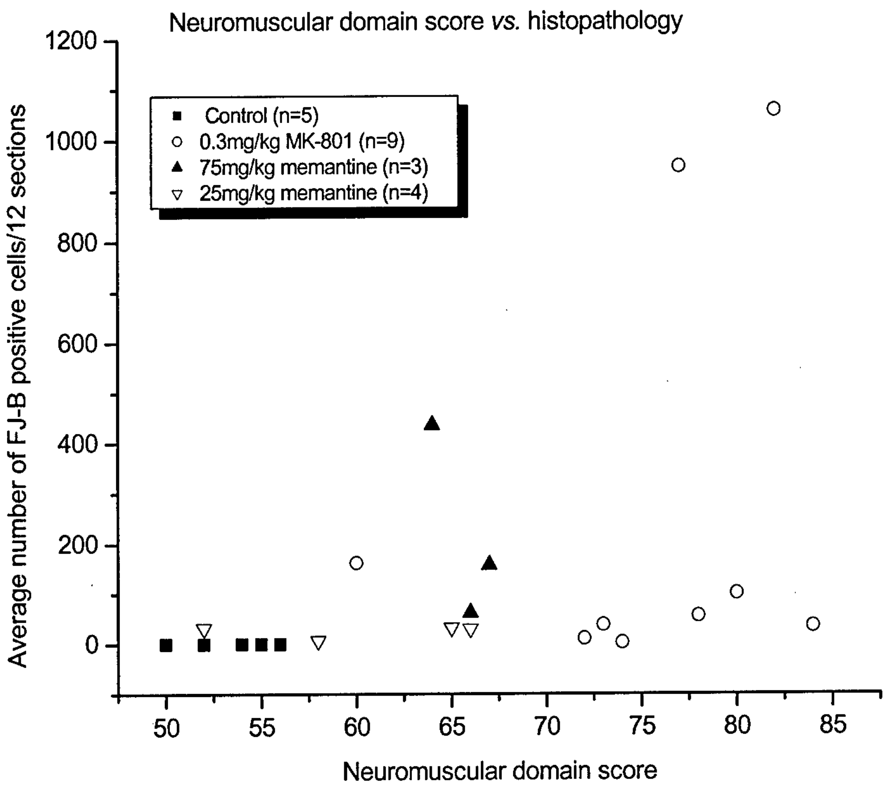
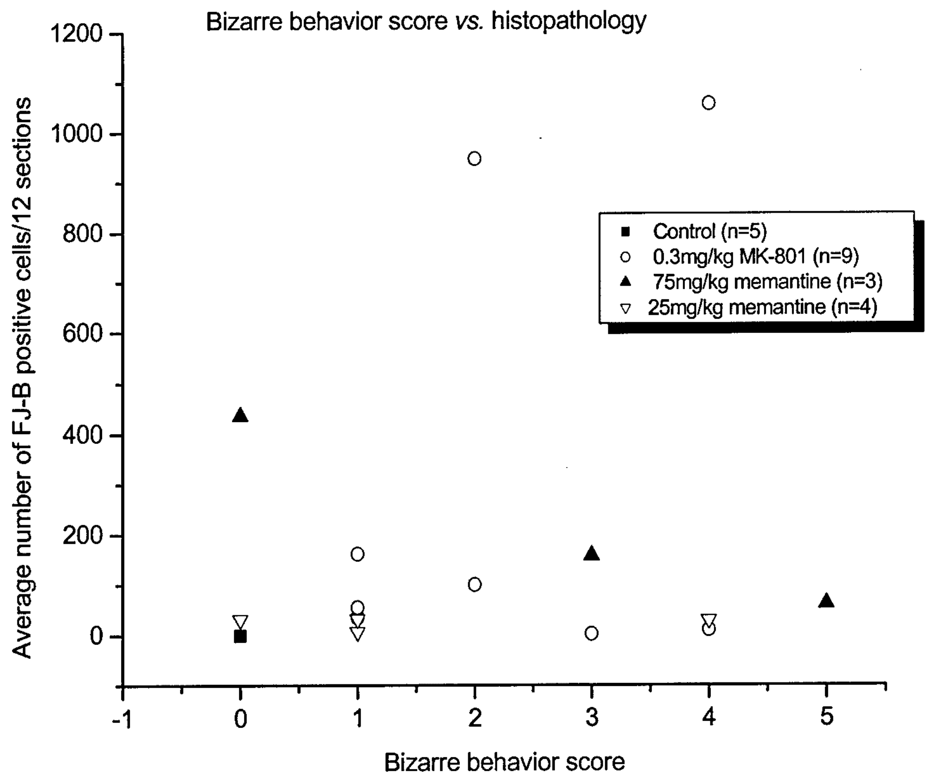

Neuromuscular domain score vs. histopathology
Control (n=5)
0.3mg/kg MK-801 (n=9)
75mg/kg memantine (n=3)
25mg/kg memantine (n=4)
Average number of FJ-B positive cells/12 sections
1200
1000
800
600
400
200
0
50
55
60
65
70
75
80
85
Neuromuscular domain score

Figure 18


Bizarre behavior score vs. histopathology
Average number of FJ-B positive cells/12 sections
1200
1000
800
600
400
200
0
-1
0
1
2
3
4
5
Bizarre behavior score
Control (n=5)
0.3mg/kg MK-801 (n=9)
75mg/kg memantine (n=3)
25mg/kg memantine (n=4)

Final discussion of *in vivo* studies:

Neurons in the PC/RSC are especially at risk to increased extracellular concentrations of glutamate, the brain's principle excitatory neurotransmitter (Olney 1989). In normal, non-pathogenic states, extracellular glutamate levels are modulated by reuptake mechanisms- even so, extracellular concentrations of glutamate are close to concentrations that also promote an excitotoxic response in susceptible neurons (reviewed by Lipton and Rosenburg, 1994). Thus, any perturbations that increase glutamate concentration within a synapse may also incite an excitotoxic response in surrounding neurons. For example, increased concentrations of glutamate (an NMDA receptor agonist) can activate surrounding NMDA receptors to such a degree that intracellular excitotoxic mechanisms may be initiated. This process has been implicated in many neurological conditions. Stroke is an example of a pathological event that leads to persistent, excess glutamate in the synapse, and may lead to subsequent cell death. Military neurotoxins that cause prolonged seizures present another situation in which endogenous glutamate can result in extensive neuronal damage and death.

When we first proposed that this neurotoxic interaction was possible, the mechanisms underlying the interaction were still speculative. However, one possible mechanism involved disinhibition. Specifically, it was possible that the two classes of drugs produced an imbalance between neuronal excitation and inhibition such that inhibition was reduced, and this resulted in excessive hyperexcitability sufficient to cause excitotoxicity in PC/RSC neurons. Olney (1991) confirmed the role the cholinergic system has in mediating glutamate release by cortical neurons, by demonstrating that the excitotoxic effect of NMDA receptor blockade can be ameliorated by the muscarinic cholinergic antagonist, atropine. Olney also determined that the muscarinic agonist pilocarpine exacerbates excitotoxicity in susceptible neurons (Corso et al., 1997). These experiments provided evidence for a complex polysynaptic mechanism of induced toxicity-through the disinhibition of excitatory glutamatergic and cholinergic pathways that converge onto pyramidal neurons located in the PC/RSC (Farber et al., 2002). This model suggests that cholinergic muscarinic receptors and glutamatergic AMPA receptors- both located on pyramidal neurons, are under inhibitory tonic control by glutamate. To maintain tonic inhibitory control over PC/RSC pyramidal neuron excitation, glutamate binds to NMDA receptors on GABAergic interneurons located in the basal forebrain and in the anterior thalamus; glutamate also binds to NMDA receptors on noradrenergic neurons that project onto basal forebrain cholinergic neurons

from the locus coeruleus. Upon NMDA receptor binding, the inhibitory neurotransmitters $GABA_A$ and noradrenaline are released onto anterior thalamus and basal forebrain interneurons. If NMDA receptors on GABAergic interneurons and on noradrenergic neurons are blocked, the release of $GABA_A$ and noradrenaline is also blocked; leaving both excitatory cholinergic and glutamatergic pathways disinhibited from releasing ACh and glutamate onto pyramidal neurons in the PC/RSC. An excitotoxic reaction then develops in pyramidal neurons as a result of their continuous depolarization (excitation) *via* muscarinic ACh and glutamatergic AMPA receptors

It is with this model in mind that we investigated the interactions of the acetylcholinesterase inhibitors (AChEI) pyridostigmine, and physostigmine with different NMDA receptor antagonists that may also be clinically encountered. Given the model of PC/RSC pyramidal innervation described above, we tested whether reduced function of NMDA receptors (by NMDA receptor antagonists) may result in excitotoxic concentrations of ACh when given in combination with AChEIs. We approached this problem using a neurotoxic screen that assesses changes in behavior, changes in motor activity and includes a histopathological analysis using the neurodegeneration marker, FJ-B.

We found that low doses of MK-801 induce neurodegeneration in the PC/RSC region of the adult female rat brain in a highly variable manner, and that the AChEI, PB ameliorates this effect (perhaps *via* binding to muscarinic receptors on GABAergic neurons). We also found that a moderate dose of physostigmine exacerbates MK-801 mediated neurodegeneration, while a low dose does not, suggesting that there is a threshold effect of ACh-mediated toxicity on pyramidal neurons specifically in the PC/RSC. We found that PB interacts with other NMDA receptor antagonists: to induce lethality in dextromethorphan co-exposed animals as well as in memantine co-exposed animals. However, in low-dose memantine co-exposed survivors, PB appears to ameliorate memantine-induced neurotoxicity, (as seen with MK-801 co-exposed animals).

Thus, our experiments suggest a dual pharmacological role for PB- it may act as a neuroprotectant against NMDA receptor antagonist-mediated neurotoxicity, since it has GABAergic properties, however, it may also be lethal in some animals co-exposed to NMDA receptor antagonists. That some animals died from co-exposure to PB and dextromethorphan while dextromethorphan exposure itself did not induce neurodegeneration in the PC/RSC

suggests that PB-induced lethality in co-exposed animals is not a result of excitotoxicity in the PC/RSC brain region, but occurs through some other mechanism.

We were surprised that animals exposed to memantine alone experienced neurodegeneration in the PC/RSC brain region. We expected that the addition of the AChEIs, PB and physostigmine would induce neurodegeneration, perhaps by exceeding a tolerable threshold of ACh in the brain. Instead we found that PB is neuroprotective (in surviving co-exposed animals), and we found no effect on neurodegeneration in low-dose memantine exposed animals given a moderate dose of physostigmine. Perhaps an excitotoxicity threshold would be exceeded given higher doses of either memantine or physostigmine. Thus, MK-801 and memantine appear to induce similar pharmacological effects either when given alone or in combination with PB, although clearly, our lowest dose of memantine was not as toxic as our low dose of MK-801. We found additional similarities between MK-801 and high-dose (75mg/kg) memantine in our behavioral studies, particularly in behaviors that measured drug-induced ataxia, hyperlocomotion, and stereotaxic behaviors- results similar to those found by other investigators.

These results are troubling because they suggest that a single therapeutic dose of memantine, a drug that has just been approved by the Federal Drug Association for the treatment of Alzheimer's Disease, can induce neurotoxic reactions in an area of the brain that is not easily studied. Therefore, the neurotoxic effects of memantine may not be fully appreciated until there is too much damage. These results are also troubling because, PB, which is not supposed to cross the blood brain barrier, has potentially lethal CNS-mediated effects in animals co-exposed to NMDA receptor antagonists.

Finally, our results fit nicely with the proposed hypothesis of an inhibitory polysynaptic pathway that requires the activation of NMDA receptors on GABAergic neurons to mediate glutamate and ACh release onto pyramidal neurons in the PC/RSC. We show that the addition of increased ACh (by physostigmine-induced inhibition of AChEI in the CNS) exacerbates MK-801 mediated neurotoxicity, supporting the hypothesis of a threshold effect of ACh on vulnerable neurons (although we did not see this effect in animals exposed to 25mg/kg memantine). We also show that the addition of PB ameliorates both MK-801 and memantine-induced neurotoxicity. This appeared at first to contradict our results with physostigmine, however, in the recent literature PB was found to bind to muscarinic receptors on GABAergic neurons

(Santos et al., 2003), to increase concentrations of the inhibitory neurotransmitter, $GABA_A$. Thus, PB may instead act to re-establish the disinhibited pathway of excitation to PC/RSC pyramidal cells, and thus must be considered a neuroprotective agent against MK-801 and memantine-mediated neurotoxicity in the PC/RSC region of the brain. Finally, we confirmed the vulnerability of pyramidal neurons in the PC/RSC region to NMDA receptor antagonist-mediated neurotoxicity. Although we looked elsewhere in the brain, particularly in the dentate and hippocampal region for induced neurotoxicity, we did not find any other affected regions.

In vitro **studies:**

Introduction:

When we began this study, the literature indicated that NMDA antagonists were neurotoxic in the cingulate/retrosplenial cortex (PC/RSC) region in the rat brain, but there was no information about how this occurred. As a result of the studies funded by this grant from the DoD, we now know one reason that these drugs are neurotoxic: They suppress inhibitory processes more in these areas than in other areas. We have shown that there is a correlation between NMDA antagonist suppression of inhibition and the level of neurotoxicity in two brain areas—the cingulate/retrosplenial cortex compared to the parietal cortex.

This is an incredibly important finding, because it alerts the scientific community to the possibility that there can be remarkable differences in the sensitivity of different brain areas to a neuroactive chemical. In this case, drugs that are supposed to be neuroprotective (NMDA antagonists) are in fact neurotoxic, simply because of the way a particular brain area is 'wired'. Thus, we can no longer assume that a drug that is neuroprotective of a particular area will be so everywhere in the brain.

As a consequence of this finding, we have moved to test the newly approved NMDA antagonist drug, memantine. The FDA has just approved this drug for Alzheimer's Disease (AD). We have just found that this drug is neurotoxic in the cingulate/retrosplenial cortex and are preparing to report this. We will do the parallel electrophysiology to confirm that memantine is suppressing inhibition.

Thus, the electrophysiological studies funded by this grant have not only resolved an important scientific issue for NMDA antagonists, but have raised the very important issue that some potential pharmaceuticals could have serious effects that may go unrecognized when used in a neuro-compromised population such as patients with AD.

I: Electrophysiological studies:

NMDA receptor antagonists disinhibit rat posterior cingulate and retrosplenial cortices: A potential mechanism of neurotoxicity.

Qiang Li, Suzanne Clark, Darrell V Lewis, and Wilkie A Wilson

This paper is included in: ***Appendix A***

II: <u>Electrophysiological studies:</u>

Differential effect of ethanol on NMDA EPSCs in pyramidal cells in the posterior cingulate cortex of juvenile and adult rats.

Qiang Li, Suzanne Clark, Darrell V Lewis, and Wilkie A Wilson

This paper is included in: ***Appendix B***

<u>Discussion</u>

These in vitro studies allowed us to begin a program of electrophysiological analysis of the inhibitory and excitatory circuits of a brain area that is sensitive to NMDA antagonists. We tested our original hypothesis that NMDA antagonists would be neurotoxic through excessive excitation, and we showed that this occurred and that it occurred because, at least in part, of the suppression of inhibition by these agents. Recall that NMDA antagonists are supposed to act by suppressing the excitatory actions of glutamate through the NMDA subtype of glutamate receptors. They do exactly that, but in the cingulate/retrosplenial cortex, those same receptors control the excitability of the inhibitory interneurons- thus suppressing these neurons is equivalent to increasing the excitability of the circuit. This finding should serve as a warning to those who would treat neurotoxin exposure with NMDA antagonists- this may result in increased damage to any area of the brain in which inhibitory drive is dependent on NMDA receptor function.

The study of this area in our lab will not stop with the ending of this grant. We demonstrated above that we have just developed the capability to record from presynaptic and postsynaptic coupled neurons in a slice preparation (figure 19). We will use this to examine in detail the effects of NMDA antagonists on synaptic inhibition.

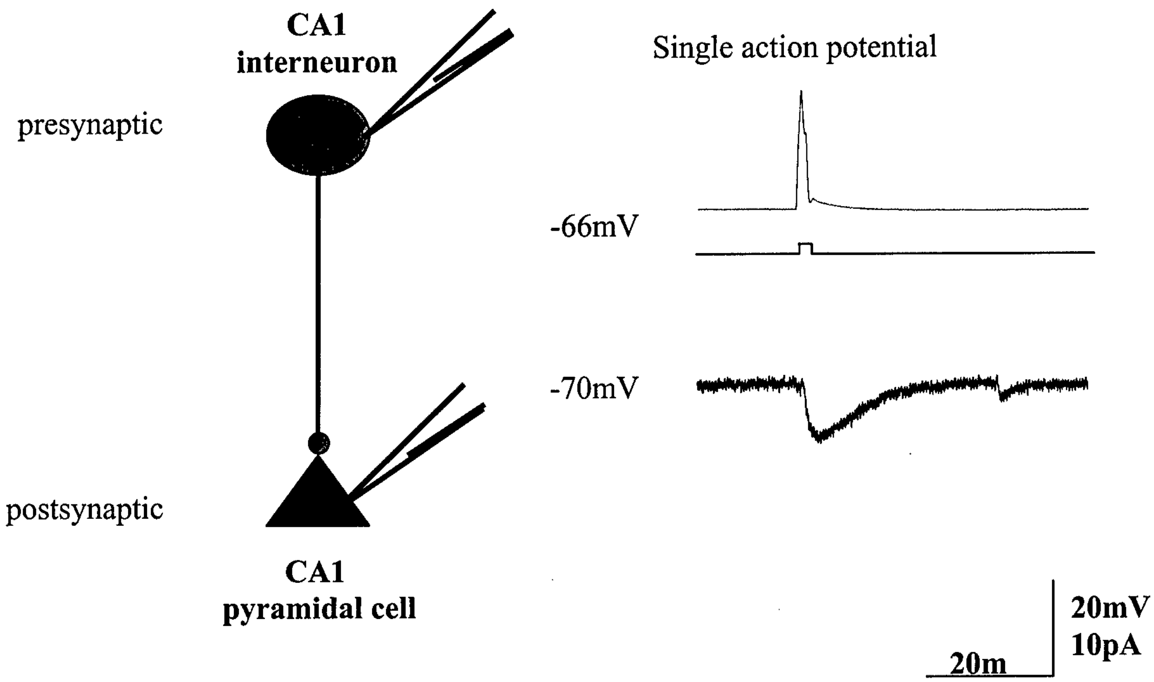
These studies have led us to apply for additional funding from non-DOD sources. Two of the junior investigators for this program, Dr. Katherine H. Jones, and Dr. Qiang Li have become intrigued with this project and have applied for and received their own funding to work

in this area. Dr. Wilson has a grant application submitted to the Veterans Administration to research related issues in deployment health.

Figure legend:

Figure 19. Schematic diagram of dual-cell recording between a pyramidal neuron and interneuron in the PC/RSC. Left panel: Schematic showing dual intracellular recordings from a presynaptic interneurons and a post-synaptic pyramidal cell in the hippocampal CA1 area. Right panel: Postsynaptic pyramidal cell held at -70mV with a pipette containing cesium chloride responded to a single action potentials (holding potential of -66mV under current-clamp condition with a pipette containing potassium, top trace), elicited by a brief positive pulse (middle trace), with a unitary postsynaptic inhibitory current (uIPSC) (bottom trace). There was a slight delay between the onset of the single action potential and the onset of the uIPSC in postsynaptic pyramidal cell. The uIPSCs were completely blocked by bath application of GABA-A receptor blocker Bicuculline (20 μM), indicating that the recorded uIPSCs were mediated by action of presynaptically released GABA on the postsynaptic GABA-A receptor.

Paired patch-clamp recordings



KEY RESEARCH ACCOMPLISHMENTS

I: *In vivo* studies:

Year one: We used histological methods to assess neurotoxicity after *in vivo* exposure to the NMDA receptor antagonist, MK-801 or to the AChEI agent, pyridostigmine bromide (PB). Specifically, we used the fluorochrome, Fluoro-jade to assess neurotoxicity in the PC/RSC in adult female rats. We found:

1. MK-801 (0.3-3mg/kg) produces neurodegeneration in the PC/RSC.
2. Given alone, PB (0.1mg/kg) does not induce neurodegeneration.

Conclusions: The Fluoro-Jade chromophore is an accurate method for detecting dying neurons induced by the prototypical NMDA receptor antagonist, MK-801 in the PC/RSC region in the rat.

Year two: We changed from the first generation Fluoro-Jade stain to Fluoro-Jade B (FJ-B), because this stain proved to be superior to the standard Fluoro-jade stain we used in our first year of study. We also began to assess the animals using a functional observation battery (FOB) prior to and after exposure to the NMDA receptor antagonists and AChEIs of interest. The results from these studies are as follows:

1. The AChEI, physostigmine does not induce neurodegeneration in the PC/RSC.
2. Physostigmine exacerbates MK-801-induced neurotoxicity at 0.3mg/kg, but not at 0.1mg/kg.

Conclusions: As reported previously, MK-801 produced neurodegeneration in the PC/RSC, whereas PB did not. Physostigmine also does not induce PC/RSC pyramidal cell neurodegeneration. At this point, we note a high degree of variability among the animals given MK-801 alone in the number of FJ-B positive cells expressed. Physostigmine produces a concentration-dependent exacerbation of MK-801-mediated neurotoxicity. Since increasing the concentration of physostigmine should also increase the concentration of available acetylcholine in the synapse, this result suggests that there is a threshold effect of acetylcholine excitation on

PC/RSC pyramidal neurons- more neurons are induced to die when they are under NMDA receptor blockade and exposed to increased acetylcholine concentrations.

Year three: The histological results from the previous two years suggested that there was some interaction between the NMDA receptor antagonist, MK-801 and the AChEIs, PB and physostigmine. At higher doses (0.3 and 1.0mg/kg), PB appeared to be neuroprotective when animals were co-exposed to 0.3mg/kg MK-801. Thus, we elected to use 1.0mg/kg PB to test potential neurotoxic (or neuroprotective) interactions with several other NMDA receptor antagonists in co-exposed animals. Physostigmine was protective against MK-801-mediated neurotoxicity at the higher dose (0.3mg/kg), but not at the lower dose (0.1mg/kg), we therefore chose to test physostigmine at 0.3mg/kg in the remaining experiments. The NMDA receptor antagonists we chose were those that may be clinically encountered, we began testing each agent at the highest proposed dose, and found the following results:

1. The NMDA receptor antagonists, dextromethorphan and felbamate do not induce neurotoxicity in exposed animals, nor does co-exposure of these compounds to pyridostigmine bromide induce detectable neurotoxicity.
2. The NMDA receptor antagonist, memantine induces a neurotoxic response visualized by positive FJ-B in mature female rats; this effect appears to be exacerbated by pyridostigmine bromide.
3. Co-exposing animals to 1.0mg/kg PB and a high (50mg/kg) dose of dextromethorphan was lethal for two of three animals tested.
4. Co-exposing animals to 1.0mg/kg PB and all proposed doses (25, 37.5, 50, and 75mg/kg) of memantine was lethal for nine of twenty-six animals tested.
5. The resultant neuropathology in MK-801 and memantine exposed animals is in good agreement with the behavioral deficits exhibited by animals exposed to these compounds.

Conclusions: Memantine is itself a neurotoxic agent to pyramidal cells in the PC/RSC. Also, the combination of the AChEI agent, PB in combination with some NMDA receptor antagonists (dextromethorphan and memantine) can prove lethal in some animals. However, none of the six animals that were co-exposed to MK-801 and PB died. Finally, our behavioral data support our histological data with respect to the neurotoxic effects of NMDA receptor antagonists on

PC/RSC neurons. Specifically, both of the non-competitive, voltage-sensitive NMDA receptor antagonists that we investigated (MK-801 and memantine) caused profound stereotypical neurobehavioral effects that usually corresponded to neurotoxic damage in the PC/RSC. These behaviors included head-weaving, and increased locomotion ("aimless wandering").

Year four: We asked for and received a one-year extension on this grant to pursue the interaction between the NMDA receptor antagonist, memantine and the peripherally acting AChEI agent, PB. Results from the past three years of *in vivo* experiments reveal some interesting points that we tried to resolve during our final year: We tried to resolve why PB appears to be protective against neurotoxicity induced by MK-801 but not with memantine, and we also tried to establish a concentration-dependent neurotoxic response to memantine by measuring neuronal degeneration in the PC/RSC using FJ-B.

1. PB (1.0mg/kg) is somewhat neuroprotective against low dose (25mg/kg) memantine-mediated neurotoxicity.
2. The average number of positive FJ-B neurons increases as the dose of memantine increases.
3. Physostigmine (0.3mg/kg) does not modulate memantine-induced neurotoxicity.

Conclusions: There is a very high degree of variability expressed among the rats in the numbers of FJ-B positive cells induced by memantine, which is similar to previous results we found with MK-801. This forced us to increase the numbers of animals we used in these last experiments, and allowed us to conclude that a 25mg/kg dose of memantine appears to be a threshold dose for inducing neurotoxicity; in some animals, there is very little damage in the PC/RSC at this dose. However, all memantine exposed animals always showed some memantine-induced neurodegeneration. Co-exposing animals to PB (1.0mg/kg) reduced the average number of memantine-induced FJ-B positive cells, and one co-exposed animal had no detectable FJ-B positive cells. Physostigmine (0.3mg/kg) also appears to reduce memantine-induced neurotoxicity. This result contrasts our results from MK-801 and physostigmine (0.3mg/kg) co-exposed animals. However, it may be that a slightly higher dose of physostigmine would exacerbate memantine-induced neurotoxicity; perhaps at 25mg/kg of memantine the "excitotoxic threshold" of acetylcholine has not been reached as it may have for a low dose of MK-801. A

low dose of MK-801 (0.3mg/kg) produces far greater numbers of FJ-B positive neurons than does a low dose (25mg/kg) of memantine, thus, the same "level" of neurotoxicity by memantine-induced NMDA receptor blockade is not reached as it is in MK-801 exposed animals. Finally, memantine-induced neurotoxicity is concentration-dependent. On average, as the dose of memantine increased, so did the numbers of positive FJ-B neurons in the PC/RSC.

II: ***In vitro* studies:** We used electrophysiological studies, including patch-clamp recordings of single neurons to test the proposed hypothesis- that NMDA receptor antagonist-induced neurotoxicity is mediated by disrupting GABAergic inhibition of principal cells in the PC/RSC. **Year one:** We examined the effects of the prototypical NMDA receptor antagonist, MK-801 on $GABA_A$ –mediated inhibitory post-synaptic currents (IPSCs) of pyramidal cells in the retrosplenial cortex (RSC). Using whole-cell patch-clamp techniques we found:

1. Bicuculline-sensitive IPSCs were recorded under bath application of MK-801 (10-40uM) at holding potentials of –5 to + 30mV. MK-801 caused a concentration dependent decrease in the frequency of spontaneous IPSCs in the majority of recorded pyramidal cells. MK-801 also reduced the amplitude of evoked $GABA_A$ receptor-mediated IPSCs in pyramidal cells recorded in layers II-VI in the RSC.
2. MK-801 has a greater inhibitory effect on pyramidal IPSCs in the RSC than in the parietal cortex.
3. Bath application of 40uM MK-801 in the presence of 0.5uM tetrodotoxin (TTX) does not influence the amplitude or frequency of mini-nature IPSCs. This suggests that the inhibitory effect of MK-801 is mediated through an action potential dependent mechanism.

Conclusions: The results from patch-clamp analysis suggest that NMDA receptors regulate GABA release. MK-801 mediated blockade of NMDA receptors causes a region-specific decrease in inhibitory synaptic transmission to pyramidal cells. This effect may be *via* presynaptic mechanisms that may suppress excitatory drive onto inhibitory interneurons to cause region-specific neurotoxicity.

Photodiode studies: We used voltage-sensitive dyes to assess acute changes in calcium concentration in the presence of MK-801 and PB. We found that a low concentration of MK-

801 (3uM) causes depolarization across cell layers in the PC/RSC, whereas PB (100uM) is inhibitory.

Conclusions: The NMDA receptor antagonist, MK-801 induces excitability in PC/RSC neurons. However, the AChEI agent, PB reduces PC/RSC excitability.

Year two: In the second year of this study, we focused on recording responses in interneurons in the PC/RSC. Interneurons were used to assess the effect of bath applied MK-801 on firing patterns. The interneurons were identified visually and by their characteristic firing patterns using patch-clamp recordings. We also examined whether cholinergic agents, specifically, the agonist pilocarpine, and the antagonist, physostigmine, influence MK-801 induced neurotoxicity in PC/RSC interneurons.

1. The non-competitive NMDA receptor antagonist, MK-801 reduces bicuculline-sensitive IPSCs at doses as low as 3uM. Again, PC/RSC pyramidal cells are more sensitive to MK-801 exposure than are pyramidal cells in the parietal cortex. The competitive NMDA receptor antagonist, APV has a similar effect as MK-801 in reducing bicuculline-sensitive IPSCs.
2. MK-801 reduced NMDA receptor-mediated excitatory post-synaptic currents (EPSCs) in interneurons in the PC/RSC.
3. The muscarinic agonist, pilocarpine and the AChEI, physostigmine reduce bicuculline-sensitive IPSCs recorded in PC/RSC pyramidal cells. This effect was additive with the effect of MK-801.
4. Ethanol blocks NMDA receptor-mediated EPSCs in PC/RSC pyramidal cells, ethanol also inhibits IPSCs more readily in brain slices from young rats than from adult rats.
5. There are age-related differences in the effect that MK-801 has on bicuculline-sensitive IPSCs in PC/RSC pyramidal cells. The time required by MK-801 to reduce the peak IPSC amplitude (to 50%) is shorter in slices from adult rats than in young rats.

Conclusions: These studies help to provide electrophysiological evidence for a complex, polysynaptic mechanism that mediates the excitation of PC/RSC pyramidal neurons. We found that MK-801 reduced NMDA receptor-mediated EPSCs in PC/RSC interneurons, providing

evidence for the first time that interneurons in this region are driven by NMDA receptor-mediated activity. This property may explain the unique sensitivity that pyramidal neurons have to NMDA antagonist induced neurotoxicity; if in the presence of an NMDA receptor antagonist, inhibitory interneurons lose their excitatory drive, and if this results in excitatory pyramidal cells losing their GABAergic synaptic inhibitory drive, the net disinhibition of excitatory neurons could allow hyperexcitability and subsequent excitotoxicity to occur. We also determined that cholinergic inputs act in the PC/RSC to mediate IPSCs; we used two different agents to increase acetylcholine concentrations using two different mechanisms and found that the muscarinic agonist pilocarpine, and the AChEI physostigmine additively reduce IPSCs in recorded PC/RSC pyramidal neurons in the presence of MK-801. Finally, older animals may be more sensitive to the neurotoxic effect of NMDA receptor antagonists. Age-related differences were found in the effects of the NMDA receptor antagonists: ethanol and MK-801; at any given dose of ethanol, NMDA-mediated EPSCs in slices from juvenile rats were more suppressed than those from adult rats, also, the time required by MK-801 to reduce bicuculline-sensitive IPSCs to 50% is shorter in slices from adult rats than from juvenile rats. These results suggest that older animals may be more sensitive than young animals to the neurotoxic effects of NMDA receptor antagonists.

Year three: These experiments focused on the effect that the NMDA receptor antagonist, memantine has on cortical pyramidal neurons using patch-clamp recordings in the presence of the AChEI, PB. In these experiments, spontaneous NMDA receptor-mediated IPSCs (sIPSCs) were isolated (using AP-5 and DNQX) and recorded in PC/RSC pyramidal neurons. We found:

1. Overall, PB (250uM) had either no effect or produced only a slight reduction in pyramidal neuron sIPSCs.
2. Memantine (300uM) either reduced recorded sIPSCs or had no effect on sIPSCs when tested alone on pyramidal cells, however, when co-applied with PB, most pyramidal neurons showed a profound reduction of sIPSCs.

Conclusions: The result of co-application of the NMDA-receptor antagonist, memantine in combination with the AChEI agent, PB is a reduction in sIPSCs by pyramidal neurons. This loss of inhibition may make vulnerable pyramidal neurons susceptible to excitotoxic mechanisms

induced by normal physiological concentrations of the neurotransmitters, acetylcholine and glutamate, because the inhibitory component onto PC/RSC pyramidal neurons is itself inhibited.

REPORTABLE OUTCOMES

Abstracts:

The following two abstracts were presented at the Society for Neuroscience Annual Meeting (Nov. 4-9, 2000).

1. MK-801, an NMDA receptor antagonist, modulates the inhibitory postsynaptic currents (IPSCs) in pyramidal neurons in the cingulate cortex of rats. Qiang Li, Suzanne Clark, Wilkie A Wilson, and Darrell V Lewis.

2. Effects of *N*-methyl-D-aspartate (NMDA) antagonists and acetylcholinesterase inhibitors (AChEIs): Fluoro-Jade staining, behavior, and photodiode array imaging. Suzanne Clark, Yolanda D Phillips, C Wang, Ashok K Shetty, Vandana Zaman, Katherine H Jones, and Wilkie A Wilson

The following abstract was presented at the Research Alcohol Association Meeting (July, 2001).

1. Effect of ethanol on NMDA receptor-mediated EPSCs of pyramidal cells in the post cingulate gyrus of juvenile and adult rats. Qiang Li, Wilkie A Wilson, and H Scott Swartzwelder.

Papers:

1. NMDA receptor antagonists disinhibit rat posterior cingulate and retrosplenial cortices: A potential mechanism of neurotoxicity. Qiang Li, Suzanne Clark, Darrell V Lewis, and Wilkie A Wilson

2. Differential effect of ethanol on NMDA EPSCs in pyramidal cells in the posterior cingulate cortex of juvenile and adult rats. Qiang Li, Suzanne Clark, Darrell V Lewis, and Wilkie A Wilson

CONCLUSIONS

Our studies were designed to determine the neurotoxic consequences of combining *N*-methyl-D-aspartate (NMDA) receptor antagonists with acetylcholinesterase inhibitors (AChEI). NMDA receptor antagonists are of great pharmaceutical interest for a wide variety of NMDA receptor-mediated neuropathologies, including acute emergencies such as stroke, or chronic illnesses such as neuropathic pain or dementias. However, there appears to be a critical balance between the effectiveness of this class of drugs in blocking the NMDA receptor, and the undesirable side effects they can induce, which include disassociative feelings and hallucinations.

Although NMDA receptor antagonists are usually only clinically encountered (with the exception of alcohol), the chance of exposure to AChEI agents may not be as predictable- for example, exposure to pesticides, or to a chemical nerve agent. In other cases, AChEI agents are prescribed, for Alzheimer's Disease, or Myasthenia Gravis, or they may be given to military personnel as a prophylactic agent against nerve gas exposure.

One hallmark of NMDA antagonist-induced neurotoxicity is the region-specific damage it produces in the brain. In the human, damage to the PC/RSC may produce significant effects on human behavior and functioning, but because those areas are involved in complex emotional processing and higher executive functions, evidence of the damage might be difficult to detect outwardly - even though the actual outcome is a profoundly impaired individual.

Although our studies were done using a rat model, the results are troubling: not only does the recently approved drug- memantine, induce neurodegeneration at doses modestly higher than the currently approved therapeutic dose, but this class of drugs (NMDA receptor antagonists) interacts with a peripherally-acting AChEI, PB to induce lethality in several co-exposed animals. Our histopathology studies confirmed the role that excess ACh may have in exacerbating the excitotoxic effects of the prototypical NMDA receptor antagonist, MK-801 (by increasing AChEI activity)- thus adding evidence to the proposed models by (Kim et al., 1999, Farber et al., 2002)- that pyramidal neurons in the PC/RSC are vulnerable to the excitotoxic effects of the neurotransmittors, glutamate and ACh. Our electrophysiological studies further confirm that pyramidal cells in the PC/RSC brain region are particularly vulnerable to the excitotoxic effects of these neurotransmitters; NMDA receptor antagonists suppress inhibition more in the PC/RSC region compared to the parietal cortex.

This model has particular significance to medical treatment of individuals in active military duty as well as veterans, as new drugs based on these mechanisms are being developed to treat Parkinson's Disease (Parsons et al., 1999) and pain (Fisher et al., 2000; Hewitt, 2000; Schmid et al.,1999), as well as many other neurological conditions that are significant for good medical care of U.S. veterans and their families. Doctors may feel pressured to prescribe these drugs by their patients or the patient's caregivers. It is important that at the very least, these doctors can approach this problem with enough information to be extra vigilant to determine whether NMDA receptor antagonists may be exacerbating neurodegeneration in areas of the brain not easily assessed.

As a result of these studies, we have submitted three abstracts, and have two published manuscripts regarding our electrophysiological data- and we are additionally preparing a third manuscript for publication regarding our histopathology data and NMDA receptor antagonist interactions with PB.

In summary, the questions we addressed in this proposal are as significant as ever, given the wider range of therapeutic drugs with potential NMDA antagonist activity, the significance of the areas of the brain affected and, unfortunately, the ever-greater risk of nerve gas exposure.

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APPENDICES

Appendix A: NMDA receptor antagonists disinhibit rat posterior cingulate and retrosplenial cortices: A potential mechanism of neurotoxicity.

Qiang Li, Suzanne Clark, Darrell V Lewis, and Wilkie A Wilson

Appendix B: Differential effect of ethanol on NMDA EPSCs in pyramidal cells in the posterior cingulate cortex of juvenile and adult rats.

Qiang Li, Suzanne Clark, Darrell V Lewis, and Wilkie A Wilson

Appendix C: MK-801, an NMDA receptor antagonist, modulates the inhibitory postsynaptic currents (IPSCs) in pyramidal neurons in the cingulate cortex of rats.

Qiang Li, Suzanne Clark, Wilkie A Wilson, and Darrell V Lewis.

Appendix D: Effects of *N*-methyl-D-aspartate (NMDA) antagonists and acetylcholinesterase inhibitors (AChEIs): Fluoro-Jade staining, behavior, and photodiode array imaging.

Suzanne Clark, Yolanda D Phillips, C Wang, Ashok K Shetty, Vandana Zaman, Katherine H Jones, and Wilkie A Wilson

Appendix E: Effect of ethanol on NMDA receptor-mediated EPSCs of pyramidal cells in the post cingulate gyrus of juvenile and adult rats.

Qiang Li, Wilkie A Wilson, and H Scott Swartzwelder.

Abstract View

DIFFERENTIAL SENSITIVITY TO MK-801 OF NMDA RECEPTORS IN PYRAMIDAL CELLS OF POSTERIOR CINGULATE CORTEX OF YOUNG AND OLD RATS

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N-methyl-D-aspartate (NMDA) receptors play an important role in synaptic transmission, brain development and neurotoxicity, and NMDA antagonists cause marked neurotoxicity in the cingulate of adult, but not infant, rats. We examined the effect of a noncompetitive NMDA antagonist MK-801 on NMDA receptor-mediated EPSCs in the cortex. Whole cell patch-clamp techniques were used to isolate NMDA receptor-mediated EPSCs in pyramidal cells of slices containing posterior cingulate cortex from young (P10-12), juvenile (P28-32) and adult (P130-140) rats. EPSCs were evoked by electrical stimuli delivered at 0.05Hz and 0.1ms duration. In the presence of DNQX (20µM) and bicuculline (20 µM), evoked NMDA receptor-mediated EPSCs were recorded from cortical slices of the three age groups and were completely blocked by D-AP5 (50µM). There were no significant differences ($p>0.05$) in EPSC reversal potentials among the three groups. When pyramidal cells were held at -30mV, the average time required to reduce the amplitude of EPSCs to 50% of control was significantly longer ($p<0.05$) in young rats than in adult rats after bath administration of 3µM MK-801. Also, after 40 minutes in MK-801, young rats showed greater MK-801-insensitive EPSC residual components when compared to adults. These results indicate that the differential age-related sensitivity to NMDA antagonist-induced neurotoxicity might be a result of developmental alteration in cortical NMDA receptors. ***Supported by Neurotoxin Exposure Treatment Research Program (NETRP), Dept. of Defense (DoD) #DAMD17-99-1-9541.***

Abstract View

EFFECTS OF *N*-METHYL-D-ASPARTATE ANTAGONISTS AND ACETYLCHOLINESTERASE INHIBITORS: FLUORO-JADE STAINING, BEHAVIOR, AND PHOTODIODE ARRAY IMAGING.

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NMDA antagonists (e.g. MK-801 & phencyclidine) produce neurotoxicity in the posterior cingulate (PC) & retrosplenial gyrus (RSG) (Olney *et al.* '89). This is exacerbated by the cholinergic agonist pilocarpine (Corsco, *et al.* '97). Do AChEIs exacerbate this neurotoxicity? Such an interaction may have clinical, environmental, and military importance.

This interaction was studied using behavioral monitoring and Fluoro-Jade (F-J) (a fluorochrome that detects degenerating neurons- including injury by NMDA antagonists (Schmued & Hopkins,'00)). Adult female rats were treated with MK-801 (0.3-3mg/kg) ± physostigmine (1mg/kg) or pyridostigmine (0.1mg/kg), monitored 3 hours, allowed to recover for 3 days, perfusion-fixed, and cryostat-sectioned (40 mm). In the PC/RSG, dose-dependent positive F-J staining was seen after MK-801 alone. Alone, AChEIs did not cause positive F-J staining and AChEIs had no effect on toxicity of high-dose MK-801. However, pyridostigmine reduced toxicity of low-dose (0.3) MK-801 (ave. F-J +cells per PC/RSG region/section: 21.7 (MK-801) *vs.* 2.2 (MK-801+pyridostigmine). Results of behavioral tests paralleled histopathology. Neurotoxic mechanisms were explored *in vitro* in PC/RSG slices using a photodiode array and voltage-sensitive dye to monitor evoked activity. In slices with PC/RSG, evoked depolarizations were enhanced by bath-applied MK-801 (10mM); AChEIs did not increase this. Thus, AChEIs have more complex interactions with NMDA antagonists than direct cholinergic agonists such as pilocarpine (but by as-yet unknown mechanisms). DAMD17-99-1-9541; Durham VAMC IMR-Clark01; NIH-NS36741.

Close

EFFECT OF ETHANOL ON NMDA RECEPTOR-MEDIATED EPSCs OF PYRAMIDAL CELLS IN THE POST CINGULATE GYRUS OF JUVENILE AND ADULT RATS. Q. Li., W.A. Wilson, and H.S. Swartzwelder. Departments of Pharmacology and Cancer Biology, Psychiatry, and Psychology, Duke University Medical Center. Neurology Research Laboratory, VA Medical Center, Durham, NC 27705 USA (Poster presented at the Research Alcohol Assn., Montreal, Quebec, July 2001).

The inhibitory effect of EtOH on NMDA receptor-mediated synaptic transmission was investigated in neocortical slices using whole cell patch clamp recording. In the presence of DNQX (20μM) and BMI (40μM), NMDA receptor-mediated EPSCs were isolated from pyramidal cells of the post cingulate gyrus in brain slices from 1 and 3 month old rats. In slices from juvenile rats, 5, 10, 30 and 60mM EtOH reduced the mean amplitude of NMDA mediated EPSCs by 11.99, 22.84, 35.75 and 46.23%, respectively (n=17). However, the same concentrations of EtOH inhibited the mean amplitude of EPSCs by only 4.03, 8.43, 16.24 and 32.62%, in slices from adult rats compared to controls (n=8). In addition, 60mM EtOH caused a significant decrease in decay times of EPSCs in juvenile rats compared to adult rats. The results indicate that EtOH reduces NMDA receptor-mediated synaptic transmission more powerfully in juvenile rats compared to adults. ***This work was supported by NIAAA Grants 11088 to HSS, and 12478 to HSS & WAW, and by VA Senior Research Career Scientist Awards to HSS & WAW.***

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NMDA Receptor Antagonists Disinhibit Rat Posterior Cingulate and Retrosplenial Cortices: A Potential Mechanism of Neurotoxicity

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NMDA receptor antagonists produce region-specific neurodegeneration by an undetermined mechanism, but one proposed mechanism involves disinhibition. In certain areas of the brain, NMDA receptors mediate excitatory drive onto inhibitory interneurons. Thus, NMDA receptor/channel antagonists may reduce inhibition (i.e., produce "disinhibition"). If a sufficient level of disinhibition is produced, enhanced vulnerability to excitotoxicity may result. Furthermore, if there are region-specific differences in NMDA antagonist-induced disinhibition, this could underlie region-specific NMDA antagonist-induced neurotoxicity. In the present study, we tested this hypothesis by exposing rat brain slices to the NMDA receptor antagonist dizocilpine maleate (MK-801) and measuring MK-801-induced disinhibition in areas of higher and lower vulnerability to neurodegeneration [posterior cingulate/retrosplenial cortices (PCC/RSC) and parietal cortex, respectively]. Using whole-cell patch-clamp techniques, bicuculline-sensitive $GABA_A$ receptor-mediated IPSCs were measured in biocytin-labeled pyramidal neurons in the PCC/RSC and parietal cortex. In the PCC/RSC, bath-applied MK-801 (10–40 μM) produced disinhibition, shown as a concentration-dependent decrease in spontaneous IPSC frequency and amplitude; MK-801 (40 μM) also reduced evoked IPSC amplitudes. In parietal cortex, MK-801 produced significantly less disinhibition. To determine whether disinhibition is caused by presynaptic or postsynaptic mechanisms, we tested the effects of MK-801 (40 μM) against miniature IPSC (mIPSC) frequency and amplitude in tetrodotoxin (TTX; 0.5 μM)-treated slices and found that MK-801 did not alter mIPSC frequency or amplitude. Taken together, these results suggest that NMDA receptors regulate activity of inhibitory interneurons and, consequently, GABA release in certain cortical areas. This region-specific reduction in inhibitory input to pyramidal cells could underlie the region-specific neurotoxicity of NMDA antagonists.

Key words: MK-801; NMDA receptor; IPSCs; disinhibition; pyramidal cells; interneurons; cingulate cortex

Neurodegeneration can result from overactivation of NMDA receptors (Rothman and Olney 1986, 1987), causing excitotoxicity proposed to be responsible for certain neurological diseases. Consequently, NMDA antagonists were screened against animal models of epilepsy (Avoli and Oliver, 1987), ischemia (Aitken et al., 1988; Ford et al., 1989; Rod and Auer, 1989), and hypoglycemia (Wieloch, 1985).

The results from these studies were perplexing, however. NMDA antagonists were not always effective (Stasheff et al., 1989; Sveinbjornsdottir et al., 1993) nor were they always neuroprotective (MacDonald et al., 1990). In fact, some antagonists actually produced region-specific neurotoxicity (Olney et al., 1989, 1991). In rats, the most affected areas were posterior cingulate cortex (PCC) and retrosplenial cortex (RSC); other areas were less sensitive (Allen and Iversen, 1990; Olney et al., 1991; Horvath et al., 1997). Pathomorphological changes varied with dose: low doses caused mitochondrial dilation, higher doses caused neuronal death (Olney et al., 1989, 1991; Fix et al., 1995; Horvath et al., 1997).

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How do NMDA antagonists produce neurotoxicity? Olney and colleagues (Olney et al., 1991; Olney and Farber, 1995; Corso et al., 1997) proposed that NMDA antagonists produce neurodegeneration by disrupting inhibition. This disinhibition could be produced directly (by disrupting the local excitatory/inhibitory network) or indirectly (by acting through modulatory neurotransmitter systems). Results from *in vivo* studies have supported Olney's hypothesis; however, no studies have directly tested this hypothesis *in vitro* in the PCC/RSC.

Using brain slices, we tested these hypotheses. We proposed that NMDA antagonists (1) reduce GABAergic inhibition in the PCC/RSC and (2) are more effective in the PCC/RSC than in less vulnerable regions, such as parietal cortex.

The first proposal is based on reports that NMDA receptors provide excitatory drive onto inhibitory interneurons in several brain areas, including the hippocampus (Grunze et al., 1996) and olfactory cortex (Schoppa et al., 1998). Thus, NMDA antagonists produce disinhibition in these areas. We extend these findings to the PCC/RSC. The second proposal is important regarding NMDA antagonist-induced neurodegeneration, because if NMDA antagonists produce more disinhibition in PCC/RSC than elsewhere, this may underlie their region-specific neurotoxicity. Finally, the use of brain slices may help to determine the level at which NMDA antagonists disrupt inhibition (i.e., in the local excitatory/inhibitory circuits, versus at the level of extrinsic modulatory inputs).

To test these proposals, we used the NMDA antagonist dizo-

cilpine maleate (MK-801) to produce disinhibition in slices of PCC/RSC and parietal cortex. Disinhibition was assessed using whole-cell patch-clamp recordings in pyramidal cells to measure $GABA_A$-mediated IPSC frequencies and amplitudes. Here we provide the first direct electrophysiological evidence that NMDA antagonists reduced inhibitory synaptic drive onto pyramidal cells in the PCC/RSC. Also, the mechanism for region-specific vulnerabilities may be explained by our results, because MK-801 produces more disinhibition in cortical areas most vulnerable to NMDA antagonist-induced neurotoxicity.

Preliminary results have been published previously in abstract form (Li et al., 2000).

MATERIALS AND METHODS

Cortical slices. Cortical slices were prepared from young, male Sprague Dawley rats (postnatal day 15–25). Rats were isoflurane-anesthetized and decapitated. The brains were quickly removed from the skulls and placed in cold (4°C) artificial CSF (aCSF) containing (in mM): 120 NaCl, 3.3 KCl, 1.23 NaH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.8 $CaCl_2$, and 10 D-glucose at pH 7.3, previously saturated with 95%O_2/5%CO_2. Coronal cortical slices (300 μm thickness) containing the PCC/RSC or the parietal cortex (Paxinos and Watson, 1986) were cut with a Vibratome (Model 752; Campden, Berlin, Germany) and incubated in a holding chamber continuously bubbled with 95% O_2 and 5% CO_2 at room temperature (22–24°C).

Whole-cell voltage-clamp recording. Our whole-cell patch-clamp techniques have been described in our previous publication (Mott et al., 1999). For recording, patch pipettes were pulled from borosilicate glass capillary tubing (1.5 mm outer diameter, 1.05 mm inner diameter; World Precision Instruments, Sarasota, FL) on a Flaming-Brown horizontal microelectrode puller (Model P-97; Sutter Instrument Co., Novato, CA). Pipettes were filled with an intracellular solution containing (in mM): 130 Cs-gluconate, 7 CsCl, 10 HEPES, 4 Mg-ATP, pH = 7.25. The quaternary lidocaine derivative QX-314 (4 mM) (Sigma, St. Louis, MO) was also included to suppress fast sodium currents. Osmolarity was adjusted to 280 mOsm. Pipette resistances generally were in the range of 4–7MΩ. Biocytin (0.3–0.4%) (Sigma) was also added to the intracellular solution for later visualization of the morphology of the recorded cells.

After >1 hr of incubation in the holding chamber, a slice was transferred to a small submersion chamber maintained at room temperature (22–24°C) and secured in place with a bent piece of platinum wire resting on the top of the slice. Individual cells were visualized using an infrared differential interference contrast Zeiss Axioskop microscope and a 40× water immersion objective. Tight seals (>1 GΩ) were obtained on pyramidal-shaped cells, and whole-cell recordings were made after rupturing the cell membrane with gentle suction. After establishment of the whole-cell recording configuration, stable long-lasting tight-seal recordings were achieved in most cases. Spontaneous and evoked IPSCs (eIPSCs) were recorded continuously using an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA). Output current signals were DC-coupled to a digital oscilloscope (Nicolet Model 410). Series resistance was monitored throughout the recordings; a cell was discarded if it changed significantly (>20% of the control). In addition, a PCM/VCR recorder (Model 400; A. R. Vetter Co, Rebersburg, PA) was used to capture all tracings of synaptic events for off-line analysis and archiving. The stored signal was further analyzed using Strathclyde Electrophysiology Software Whole Cell Program (courtesy of Dr. John Dempster, University of Strathclyde, Glasgow, UK) with an interface (BNC-2090; National Instruments, Austin, TX) to a PC-based computer.

Cortical interneurons were selected on the basis of the shape of the soma (small and round under DIC microscopy) and their characteristic firing pattern. To determine the firing pattern of a cortical interneuron while achieving the tight seal offered by Cs-gluconate solution under the voltage clamp, the tip of a patch pipette was filled with a solution containing K-gluconate (in mM: 130 K-gluconate, 7 KCl, 10 HEPES, 4 Mg-ATP, and 0.3 Tris-GTP, pH 7.25), then backfilled with a solution containing Cs-gluconate (see above). The dialysis of the recorded cell with the Cs-gluconate solution could be observed from the distortions of shapes of the action potentials ~10 min after whole configuration was established. NMDA receptor-mediated EPSCs were then recorded from interneurons.

Electrical stimulation. A monopolar tungsten electrode (A-M System, Carlsborg, WA) was placed ~50–70 μm lateral to the soma of the recorded pyramidal cells in the same layer. The stimulus threshold was first determined by increasing the intensity of the rectangular wave pulse until detectable responses occurred. Then constant current rectangular stimulus pulses 50% higher than threshold intensity with a duration of 0.1 msec and interval of 0.0166 Hz were delivered through the electrode by an isolated stimulator (Grass S88; Grass Instruments, Quincy, MA).

Histological identification of pyramidal cells. During recording, pyramidal cells were filled with biocytin. After the end of the recording, the slice was allowed to stay in the recording chamber for an additional 10–20 min for further biocytin transport within the axon. The slices were then placed overnight in 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M PBS. The slices were washed thoroughly in PBS and incubated in 0.1 M Tris-buffered saline (TBS) containing 1% H_2O_2 for 30 min. The slices were then incubated overnight at 4°C with avidin–biotin–peroxidase complex (ABC kit; Vector Labs, Burlingame, CA) in TBS containing 0.05% Triton X-100. The slices were then rinsed three times in PBS, reacted in a solution containing 3,3′-diaminobenzidine (DAB kit, Vector Labs), then cleared and mounted. The morphology of the biocytin-filled pyramidal cells was examined with a light microscope, and cells were drawn using a camera lucida.

Statistical analysis of data and drug application. Data were analyzed off-line using Strathclyde Electrophysiological Software. The Kolmogorov–Smirnov (K-S) statistical test was used to compare two different cumulative distributions using Origin 5.0 for Windows (MicroCal Software, Norththampton, MA). Paired and unpaired *t* tests and one-way ANOVA tests were also used, when appropriate. All group data are presented as mean ± SEM.

MK-801 and TTX were purchased from RBI (Natick, MA). D-(-)2-amino-5-phosphonovaleric acid (D-AP5) and bicuculline methiodine (BMI) were purchased from Sigma. All drugs were dissolved directly in the aCSF and bath-applied in the perfusion medium for ~20 min, unless noted otherwise.

RESULTS

Pyramidal cells were recorded in layers II–VI of the neocortex. We investigated these cortical strata in the PCC/RSC and the parietal cortex. The location of the PCC/RSC and the parietal cortex are illustrated in Figure 1*A*. Data were acquired from 54 pyramidal cells, 6 interneurons in the PCC/RSC, and 49 pyramidal cells from the parietal cortex. According to the location of their somata, pyramidal cells are divided into three subgroups: the superficial layer (II–III), layer IV, and the deep layer (V–VI). In the PCC/RSC, 25 (46%) pyramidal cells are in the superficial layer, 13 (24%) in layer IV, and 16 (30%) in the deep layer. In parietal cortex, 20 (40%) pyramidal cells are in the superficial layer, 14 (29%) in layer IV, and 15 (31%) in the deep layer. All six interneurons are recorded from PCC/RSC layers II and III. Because of the irreversible blockade of NMDA receptors by MK-801, only a single experiment was done from each cortical slice treated with MK-801.

Morphology of dendritic and axonal arbors of pyramidal cells in the PCC/RSC

The morphology of each recorded cell was assessed with biocytin staining to unambiguously distinguish pyramidal cells from other cell types. We were able to recover histologically ~90% of all recorded cells in the PCC/RSC areas. The dendritic arbor of pyramidal cells in the PCC/RSC was characterized by a long apical dendrite that usually extended toward the pial surface, where typically it branched extensively to form multiple small terminal tufts just under the pial surface. Along the length of the apical dendrites are numerous obliquely branched dendritic collaterals. Basal dendrites extended outward from the lower portion of the soma; these basal dendrites ascended or descended with gradual tapering. The pyramidal cell axonal arbor was densely

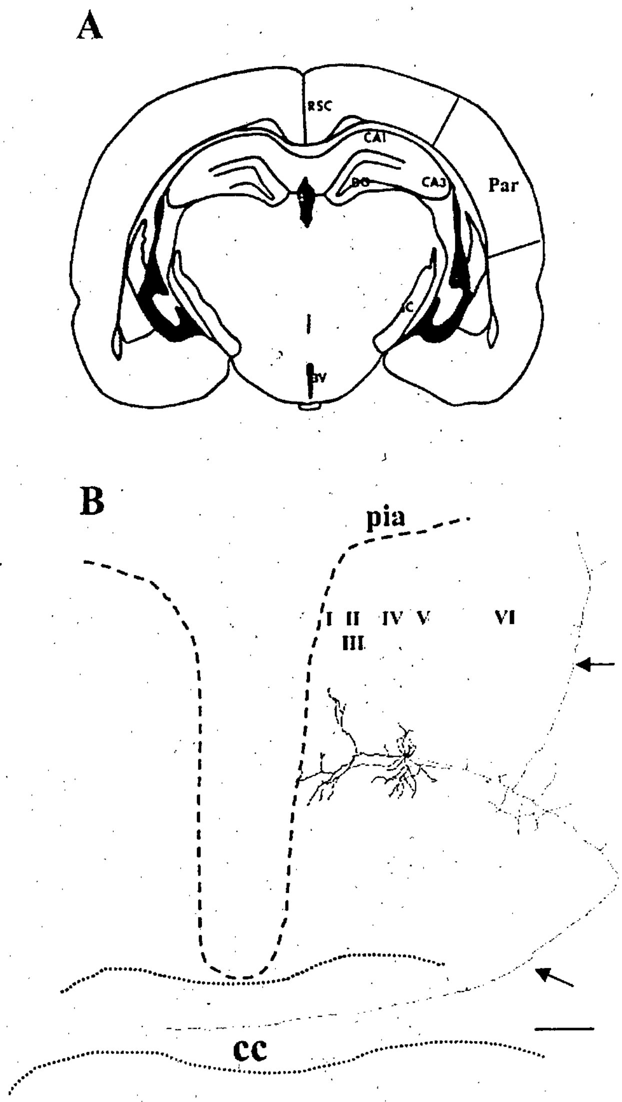

Figure 1. Camera lucida reconstruction of a PCC/RSC pyramidal cell filled with biocytin. *A*, Coronal section of a cortical slice containing PCC/RSC and parietal cortex. *B*, Camera lucida reconstruction of a biocytin-filled, layer V pyramidal cell recorded in the PCC/RSC. This pyramidal cell was filled with biocytin during the experiment. An apical dendrite arising from the soma extends toward the pial surface and branches off to form multiple tufts. In addition to a typical apical dendrite tree, a long axon originating from the soma projects to the contralateral side of the hemisphere through the corpus callosum. Another branch of the axon projects ipsilaterally to the lateral cortex. The pia is near the end of the apical dendrite branches. *Dashed lines* represent the approximate pial surface of the cortex. *RSC*, Retrosplenial cortices; *Par*, parietal cortex; *CC*, corpus callosum; *I–VI*, cortical lamina; *DG*, dentate gyrus; *3V*, third ventricle; *CA*, cornus ammonis. *Arrows* denote axon. Scale bar, 200 μm.

distributed around the soma and also extended widely, both horizontally and vertically. These morphological characteristics are consistent with cortical pyramidal cells described elsewhere (Kim and Connors, 1993; Lubke et al., 1996; Reyes and Sakmann, 1999; Feldmeyer and Sakmann, 2000). Figure 1*B* shows a camera lucida reconstruction of a PCC/RSC pyramidal cell. The soma of this pyramidal cell is located in layer V, and it sends a long axon projecting to the contralateral side across the corpus callosum (Sripanidkulchai and Wyss, 1987).

MK-801 decreases the spontaneous IPSC frequency in pyramidal cells of the PCC/RSC

Spontaneous IPSCs (sIPSCs) were recorded from pyramidal cells in the PCC/RSC using whole-cell voltage-clamp techniques. The holding potentials were +10 or +30 mV, and the calculated E_{CL} was approximately −40 mV for the internal solution used. Under these experimental conditions, the sIPSCs were inward currents at a holding potential of −70 mV (Fig. 2*A*) and were robust outward currents at a holding potential of +30 mV (Fig. 2*B*). At the end of the experiment, the recorded sIPSCs were abolished by bath application of a selective $GABA_A$ receptor antagonist, BMI (20 μM) (Fig. 2*C*). NMDA receptor-mediated EPSCs might be present when a cell was depolarized to +40 mV (Hestrin, 1992). Accordingly, we also assessed whether NMDA receptor-mediated EPSCs were also present when a cell was held at +30 mV without blocking excitatory transmissions. We examined eight PCC/RSC pyramidal cells in the presence of BMI (20 μM). When the holding potential was held at −70 mV, fast inward currents mediated by AMPA receptors dominated, and no NMDA receptor-mediated EPSCs were observed (Fig. 2*D*). NMDA receptor-mediated EPSCs only become detectable after the holding potential was changed to +30 mV (data not shown). However, long slow outward currents were recorded in only four of the nine cells tested, and the frequency of recorded NMDA–EPSCs is very low. These data indicated that the contribution of NMDA receptor-mediated EPSCs was insignificant and negligible in cells held at +30 mV. Figure 2, *E* and *F*, shows evoked IPSCs recorded in a PCC/RSC pyramidal cell in the presence of DNQX (20 μM) and D-AP5 (50 μM). A plot of the amplitude of evoked IPSCs against holding potentials (Fig. 2*E*) indicated an x-intercept of −39 mV, closely approximating the calculated reversal potential of E_{CL} (Fig. 2*F*). These results indicated that the recorded IPSCs were mediated by the activation of $GABA_A$ receptors.

Effects of MK-801 on sIPSCs in PCC/RSC pyramidal cells are demonstrated in Figures 3 and 4. MK-801 suppressed two IPSC properties: amplitude and frequency (the latter reflected as an increase in the interval between sIPSCs). Figure 3 shows recordings from a layer V pyramidal cell. At a holding potential of +30 mV, the recorded IPSCs were outward currents (Fig. 3*A*, *top panel*). Bath application of the NMDA antagonist MK-801 (40 μM) caused a significant decrease in the frequency and amplitude of sIPSCs in this pyramidal cell (Fig. 3*A*, *second panel* from *top*). The IPSCs were abolished by bath-applied BMI (20 μM) and recovered partially on washout (Fig. 3*A*, *bottom two panels*). The cumulative probability distributions for these changes are shown in Figure 3*B*. As shown, bath-applied MK-801 produced a rightward shift in the distribution of sIPSC intervals (Fig. 3*B*, *left panel*), indicating a decrease in sIPSC frequency (K-S test; $p < 0.01$). In addition, sIPSC amplitudes were also reduced significantly (K-S test; $p < 0.001$), as shown in Figure 3*B* (*right panel*). The morphology of this pyramidal cell is shown in Figure 3*C*.

This effect was not limited to layer V pyramidal cells. A similar effect was also seen in a layer III pyramidal cell of the PCC/RSC, as shown in Figure 4. This pyramidal cell responded to bath-applied MK-801 (40 μM) with a significant decrease in sIPSC frequency (Figs. 4*A*, 5*B*). In addition, amplitudes of sIPSCs were also attenuated by MK-801. Although large-amplitude sIPSCs apparently dominated in this layer III pyramidal cell, the morphology of this pyramidal cell, as shown in Figure 4*D*, is similar

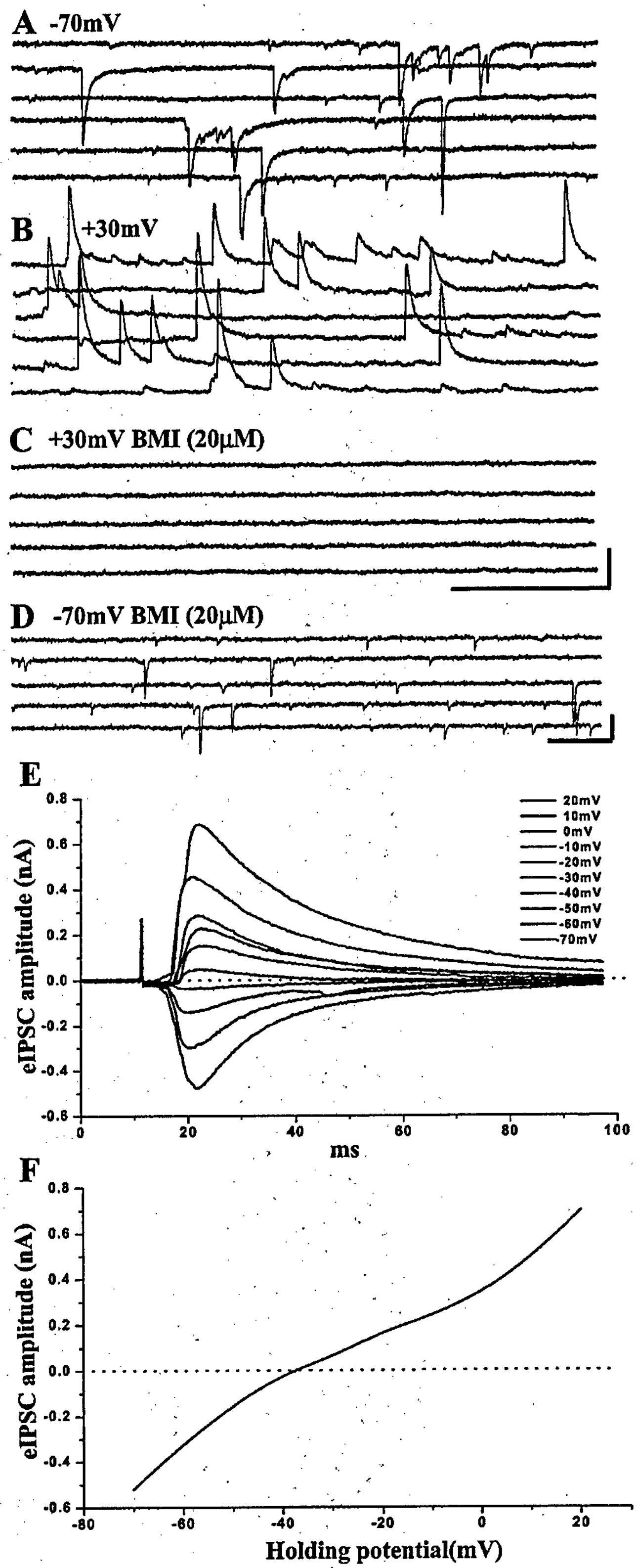

Figure 2. $GABA_A$ receptor-mediated IPSCs recorded in PCC/RSC pyramidal cells. Whole-cell recordings were performed using a CsCl-based internal solution at holding potentials of −70 mV and +30 mV. When the holding potential was −70 mV (*A*), inward currents were recorded. When the cell was held at +30 mV, the currents became outward (*B*). The $GABA_A$ receptor antagonist BMI (20 μM) abolishes the currents (*C*). In the presence of BMI (20 μM) and at the holding potential of −70 mV, fast inward currents are recorded from another pyramidal cell and can be blocked by DNQX (20 μM) (*D*). In the presence of D-AP5 (50 μM) and DNQX (20 μM), amplitudes of evoked IPSCs recorded from another pyramidal cell held at potentials ranged from −70 to +20 mV (*E*). An *I–V* curve was constructed (*F*) based on the evoked synaptic responses shown in E. The apparent reversal potential was approximately −39 mV. These findings indicate that the recorded IPSCs were mediated by $GABA_A$ receptors. Calibration: *A–C*, 500 msec, 0.1 nA; *D*, 100 msec, 50 pA.

to that of the pyramidal cell illustrated in Figure 4*C*. We found similar MK-801-mediated effects in layers II–VI as well.

Inhibitory effects of MK-801 on sIPSCs of pyramidal cells were compared among the three groups. Bath-applied MK-801 (40 μM) reduced sIPSC frequency by 43.3 ± 3.8% ($n = 16$) compared with the control in the superficial group (layers II and III), 44.4 ± 4.6% ($n = 11$) in layer IV, and 44 ± 3.0% ($n = 13$) in the deep layer (layers V and VI). There are no significant differences in mean IPSC frequency among three groups ($p > 0.05$; one-way ANOVA), so we pooled data obtained from pyramidal cells recorded in PCC/RSC layers II–VI. Overall, bath application of MK-801 (40 μM) reduced the frequency of sIPSCs by 43.8 ± 2.2% ($n = 40$) relative to control ($p < 0.05$; paired *t* test).

We also examined the concentration dependence of MK-801-induced decreases in the frequency of sIPSCs using three concentrations of MK-801 bath-applied in sequence. We monitored sIPSCs for ~20 min after each concentration was applied. Overall, reductions of mean inter-event intervals were 15.4 ± 1.4, 28.6 ± 2.3, and 44.3 ± 3.1% after bath application of MK-801 at the doses of 10, 20, and 40 μM, respectively ($p < 0.05$; one-way ANOVA; $n = 4$).

We also tested the effect of the widely used competitive NMDA receptor antagonist D-AP5. Similar to MK-801, D-AP5 (50 μM) significantly (K-S test; $p < 0.05$) decreased the frequency of sIPSCs in two PCC/RSC pyramidal cells tested (data not shown).

MK-801 reduced the amplitude of evoked IPSCs in pyramidal cells in the PCC/RSC

In these experiments, MK-801 was also tested against evoked IPSCs of PCC/RSC pyramidal cells. The pyramidal cells were held at −5 or +5mV, and an eIPSC was elicited by a single pulse delivered lateral to the recording electrode (see Materials and Methods). These eIPSCs were abolished by bath application of $GABA_A$ receptor antagonist BMI (20 μM), indicating that eIPSCs are mediated by $GABA_A$ receptors (Fig. 5*A*).

The time course of the MK-801-induced effect on eIPSC of a layer V PCC/RSC is shown in Figure 5*B*. As shown, the evoked IPSCs were gradually, but significantly, reduced by bath-applied MK-801. At a holding potential of +5mV, recordings were made in control solution for 10 min, then MK-801 (40 μM) was bath-applied. Five minutes after addition of MK-801, the eIPSCs began to decline. By 30 min after treatment, eIPSCs were reduced from 1.0 to 0.5 nA (i.e., 50% of control). Again, the eIPSCs were blocked by bath-applied BMI (20 μM) and recovered during BMI washout. Overall, MK-801 (40 μM) significantly reduced evoked responses to 51.3 ± 2.3% of control ($n = 13$; $p < 0.05$; paired *t* test).

Comparison of effects of MK-801 on sIPSCs and eIPSCs in the PCC/RSC and the parietal cortex

Several *in vivo* animal studies have demonstrated that pathomorphological changes induced by NMDA antagonists occur in a

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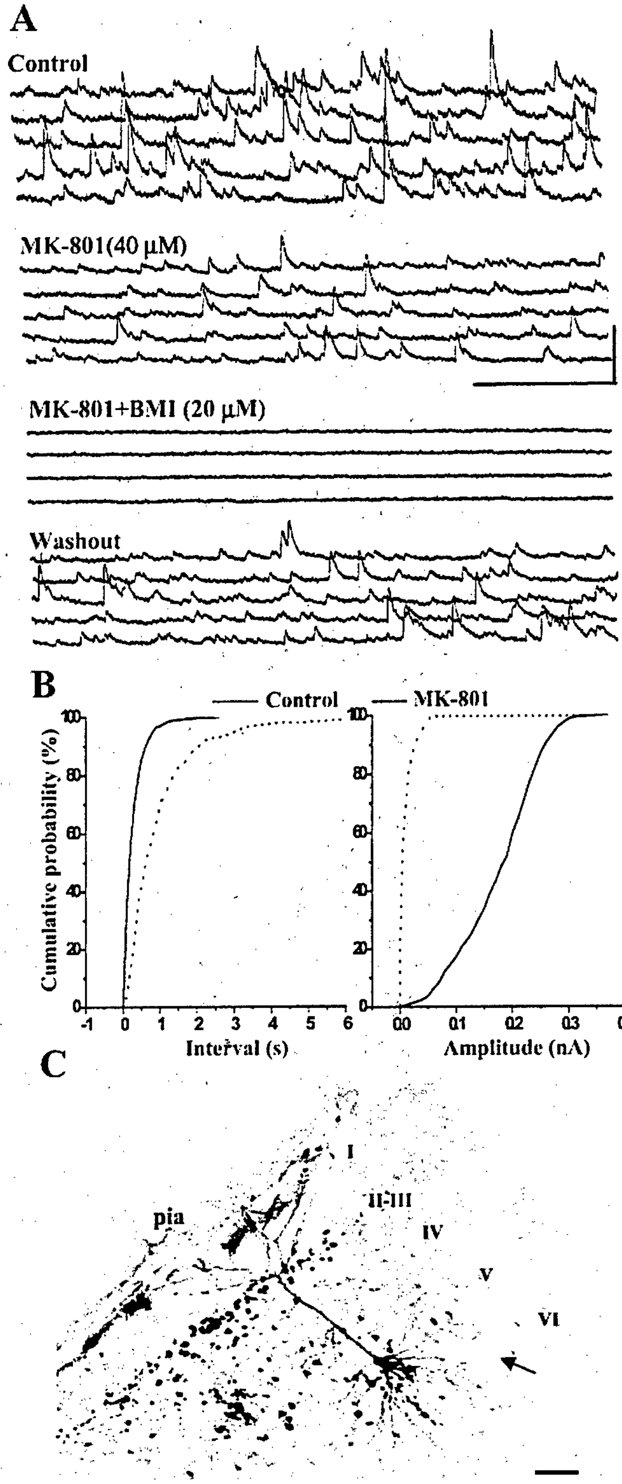

Figure 3. MK-801 decreases $GABA_A$ receptor-mediated sIPSCs. *A*, In a layer V PCC/RSC pyramidal cell held at +30 mV, bath-applied MK-801 (40 μM) decreased the frequency and amplitude of sIPSCs. When BMI (20 μM) was added, sIPSCs were blocked reversibly. Calibration: 500 msec, 0.1 nA. *B*, In the same cell, the cumulative inter-event interval distribution shows a significant increase in the inter-event interval caused by MK-801 ($p < 0.01$; K-S test). The cumulative amplitude distribution also shows a significant decrease in amplitude ($p < 0.001$; K-S test). *C*, Photomicrograph of the same cell filled with biocytin. *I–VI*, Cortical lamina. *Arrow* denotes axon. Scale bar, 100 μm.

number of different areas of the brain, but with different sensitivities among the affected areas (Olney et al., 1991; Fix et al., 1993; Horvath et al., 1997). For example, MK-801 causes more severe damage to neurons in the PCC/RSC, whereas some other cortical areas were less affected.

We hypothesize that one mechanism by which MK-801 produces region-specific neurodegeneration is that MK-801 more effectively produces disinhibition in a vulnerable area (e.g., PCC/RSC) than a less vulnerable area (e.g., parietal cortex). To address this, we recorded sIPSCs and eIPSCs from 49 pyramidal cells in the parietal cortex.

Figure 6 shows the effect of MK-801 on sIPSCs recorded in a layer V parietal pyramidal cell. The sIPSCs were only slightly reduced by MK-801 (40 μM) (Fig. 6*A*). Although MK-801 significantly increased the inter-event intervals (K-S test; $p < 0.05$) (Fig. 6*B*, *left panel*), MK-801 did not have a significant effect on the cumulative amplitude distribution (K-S test; $p > 0.05$) (Fig. 6*B*, *right panel*).

Depressive effects of MK-801 on pyramidal cell sIPSCs were also compared among the three groups. Bath-applied MK-801 (40 μM) reduced frequency of sIPSCs by 21.9 ± 4.8% ($n = 14$) compared with control in the superficial group (layers II and III), 23.9 ± 5.4% ($n = 12$) in layer IV, and 23.4 ± 3.5% ($n = 13$) in the deep layers (layers V and VI). No significant differences in mean IPSC frequency among three groups were observed ($p > 0.05$; one-way ANOVA). Accordingly, we also pooled data obtained from pyramidal cells recorded in layers II–VI of parietal cortex. Bath application of MK-801 (40 μM) reduced the frequency of sIPSCs by 22.9 ± 2.6% ($n = 39$) relative to control ($p < 0.05$; paired t test). In contrast, in the PCC/RSC, mean sIPSC frequency was reduced by 43.8 ± 2.2% ($n = 40$). In addition, we found that in ~19% pyramidal cells recorded in parietal cortex, mean sIPSC frequency was reduced by MK-801 (40 μM) <10% (ranging from 2 to 10%), compared with 5% of those in the PCC/RSC.

Overall inhibitory effect of MK-801 on sIPSC frequency of each group recorded from two brain regions is summarized in Figure 7. There is a significantly greater depression of sIPSC frequency by MK-801 in the PCC/RSC versus the parietal cortex (unpaired t test; $p < 0.05$) (Fig. 7*A*). The amplitudes of IPSCs are also suppressed by MK-801 in the PCC/RSC. Taken together, these results indicate that, at a dose of 40 μM, MK-801 caused greater disinhibition of pyramidal cells in the RSC than in the parietal cortex.

Similar differences were also observed on eIPSCs of pyramidal cells in the parietal cortex as shown in Figure 7*B*. The mean amplitude of eIPSCs of pyramidal cells recorded from the PCC/RSC was decreased by 52 ± 1.8% ($n = 10$) of control. However, the average eIPSCs amplitude of pyramidal cells recorded from the parietal cortex was decreased by only 27 ± 2.1% ($n = 9$) of control after bath application of 40 μM MK-801. There is a significant difference between two groups ($p < 0.05$; unpaired t test).

MK-801 inhibits NMDA receptor-mediated EPSCs in PCC/RSC interneurons

It has been demonstrated that excitability of interneurons in the entorhinal cortex (Jones and Buhl, 1993) and auditory cortex (Bandrowski et al., 2001), the olfactory bulb (Schoppa et al., 1998) of rats can be modulated via excitatory synaptic input mediated by NMDA receptors. To assess whether MK-801 has a direct effect on NMDA receptor-mediated EPSCs in GABAergic interneurons, we isolated NMDA receptor-mediated EPSCs

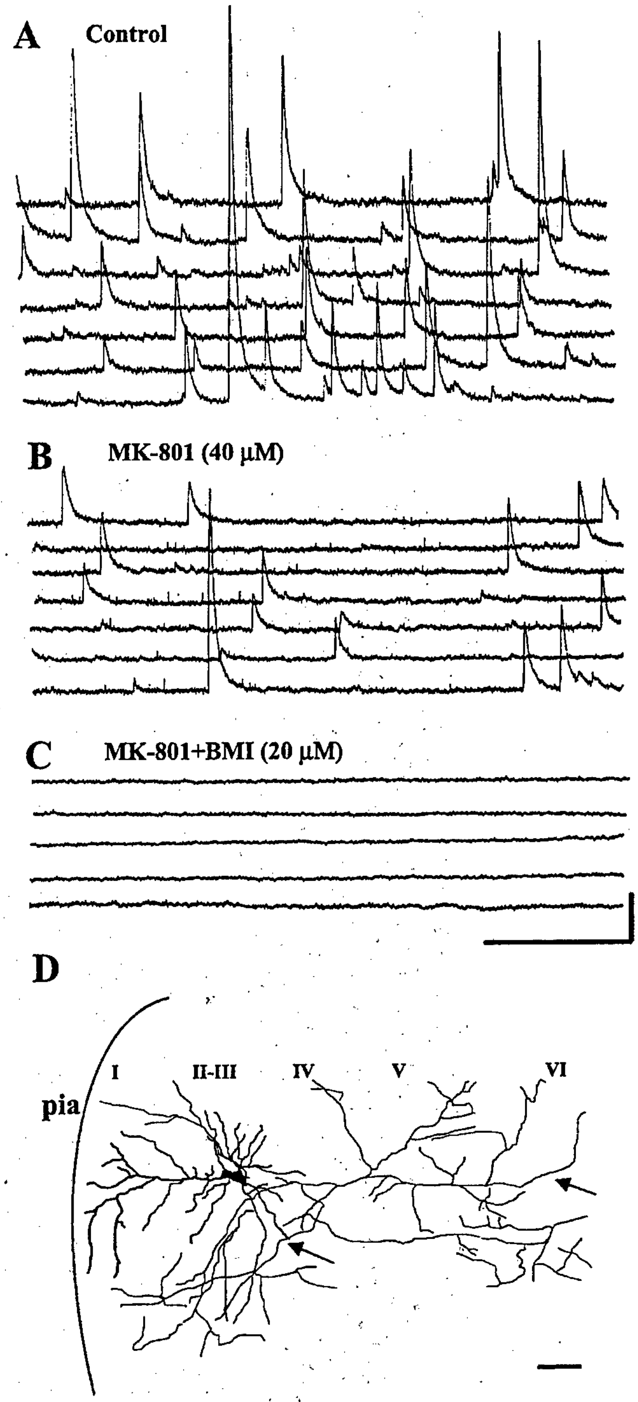

Figure 4. Effect of MK-801 on sIPSCs from a layer III cell. In a layer III PCC/RSC pyramidal cell held at +30 mV, bath-applied MK-801 (40 μM) (*B*) decreased sIPSCs compared with control (*A*), an effect similar to that seen in layer V (Fig. 3). Recorded sIPSCs were abolished by BMI (20 μM) (*C*). Calibration: 500 msec, 0.1 nA. *D*, Camera lucida reconstruction of the same cell. As shown, this is also a pyramidal cell, with processes slightly different from the cell shown in Figure 3, although the pharmacological responses to MK-801 were similar. *I–VI*, Cortical lamina. *Arrows* denote axon. Scale bar, 100 μm.

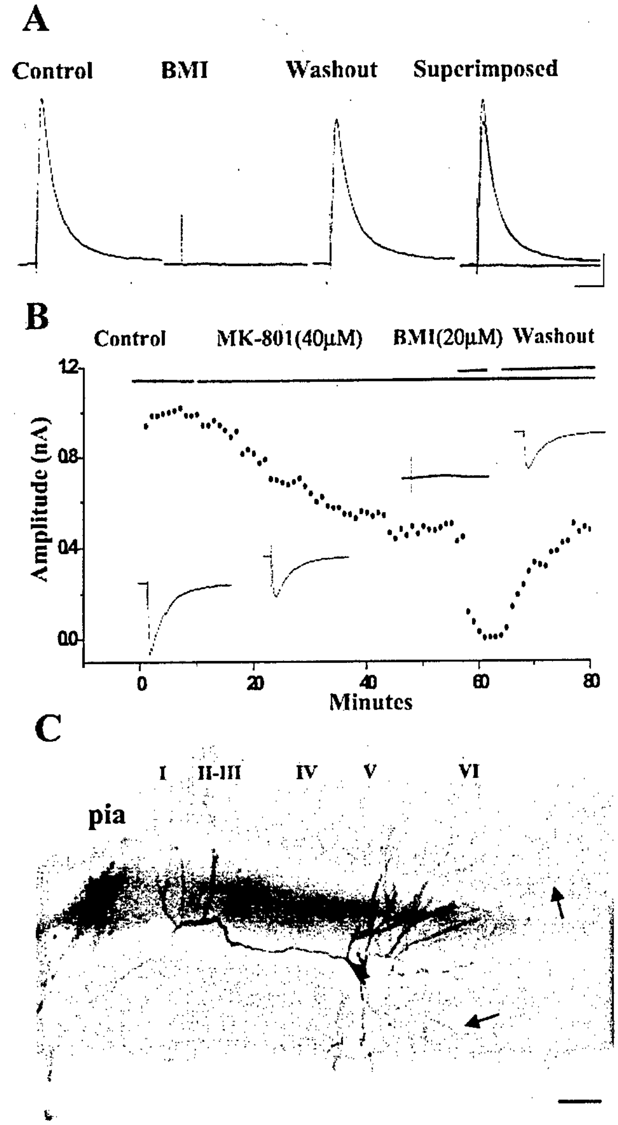

Figure 5. Effect of MK-801 on eIPSCs. *A*, In a layer IV PCC/RSC pyramidal cell held at +5mV, outward eIPSCs were recorded. These eIPSCs were completely and reversibly blocked by BMI (20 μM), as was seen with sIPSCs (Fig. 2*A–C*), indicating that eIPSCs were mediated by $GABA_A$ receptors. Calibration: 50 msec, 0.1 nA. *B*, In a layer V PCC/RSC pyramidal cell held at +5mV, the eIPSC time course after bath-applied MK-801 (40 μM), BMI (20 μM), and washout is shown. *Insets* illustrate the averaged peak IPSC traces corresponding to each treatment. *C*, Photomicrograph of the pyramidal cell shown in *B* filled with biocytin. *I–VI*, Cortical lamina. *Arrow* denotes axon. Scale bar, 100 μm.

from six PCC/RSC layer II–III interneurons. Figure 8*B* shows a typical firing pattern of a PCC/RSC layer III interneuron (Kawaguchi. 1995; Zhou and Hablitz, 1998) compared with that of a PCC/RSC layer IV pyramidal cell (Fig. 8*A*). When the interneuron was held at −70 mV, DNQX-sensitive or AMPA/kainate receptor-mediated fast EPSCs were dominant (Fig. 8*C*). When the holding potential was changed to +50 mV, there were few, very long slow outward currents (Fig. 8*D*). These could be blocked by D-APV (n = 2; data not shown), suggesting they are NMDA receptor mediated.

Twenty minutes after bath-applied MK-801 (40 μM), these

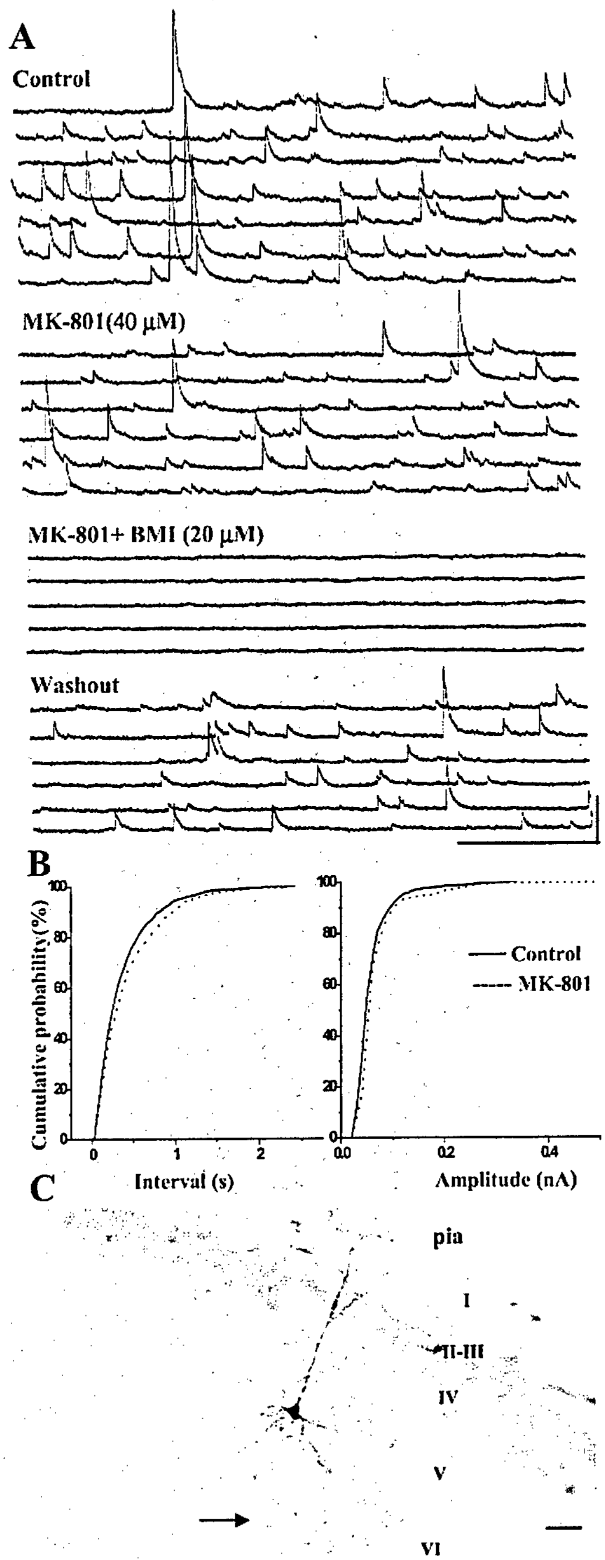

Figure 6. MK-801 is less effective on sIPSCs of pyramidal cells in the parietal cortex. *A*, At the holding potential of +30 mV, bath-applied MK-801 (40 μM) decreased sIPSCs in a layer V pyramidal cell in the parietal cortex. When BMI (20 μM) was added, sIPSCs were abolished, then recovered after washout. Calibration: 500 msec, 0.1 nA. *B*, In the same cell, the cumulative inter-event interval distribution shows a slight but significant increase in the inter-event interval ($p < 0.05$; K-S test). In contrast, MK-801 did not significantly decrease the sIPSC amplitude in the parietal cortex, as shown by the cumulative amplitude distribution ($p > 0.05$; K-S test). *C*, Photomicrograph of the same cell filled with biocytin. *I–VI*, Cortical lamina. *Arrow* denotes axon. Scale bar, 100 μm. (In the main dendrite, the small apparent gaps are artifacts: they arose after coverslipping. During the experiment, the dendrite was intact, as in all other experiments.)

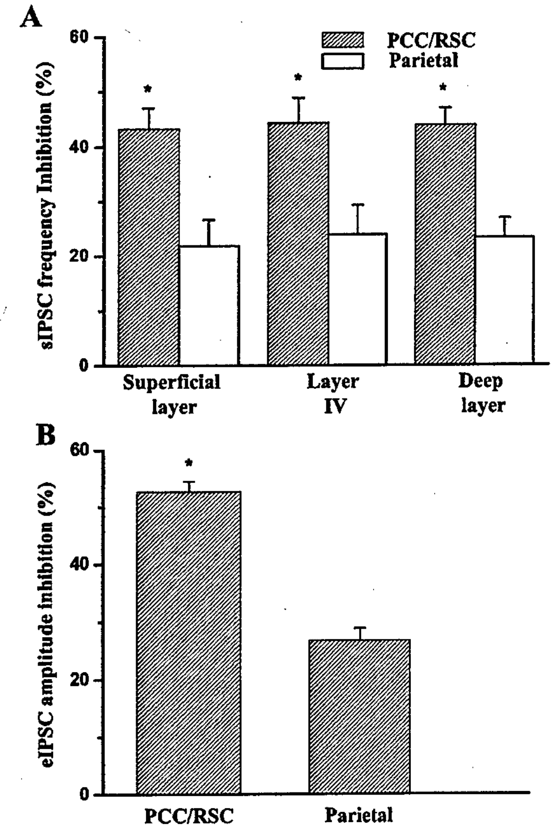

Figure 7. Comparison of MK-801 on sIPSCs and eIPSCs of pyramidal cells in the PCC/RSC and the parietal cortex. *A*, Graph shows data indicating the reduction in sIPSC frequency in PCC/RSC pyramidal cells [superficial layers (n = 16), layer IV (n = 11), and the deep layers (n = 13)] and in the parietal cortex [superficial layer (n = 14), layer IV (n = 12), and the deep layers (n = 13)]. MK-801 (40 μM) significantly decreased the average sIPSC frequency of pyramidal cells in the PCC/RSC compared with those in the parietal cortex (*$p < 0.05$; unpaired t test). *B*, Graph shows that MK-801 (40 μM) significantly inhibited the average peak amplitude of eIPSCs in pyramidal cells (n = 10) in the PCC/RSC compared with the parietal cortex (n = 9) (*$p < 0.05$; unpaired t test).

NMDA receptor-mediated EPSCs were abolished (Fig. 8*E*). MK-801 completely blocked NMDA receptor-mediated EPSCs in all six PCC/RSC interneurons tested. These results suggested that PCC/RSC interneurons receive NMDA receptor-mediated input; the absence of excitatory afferent drive onto the PCC/RSC interneurons could result in a decrease of inhibitory transmission from interneurons onto pyramidal cells.

MK-801 did not inhibit mIPSCs of pyramidal cells in the PCC/RSC

The results described above demonstrated that a block of NMDA receptors by the NMDA antagonist MK-801 reduced $GABA_A$ receptor-mediated IPSCs in PCC/RSC pyramidal cells. To de-

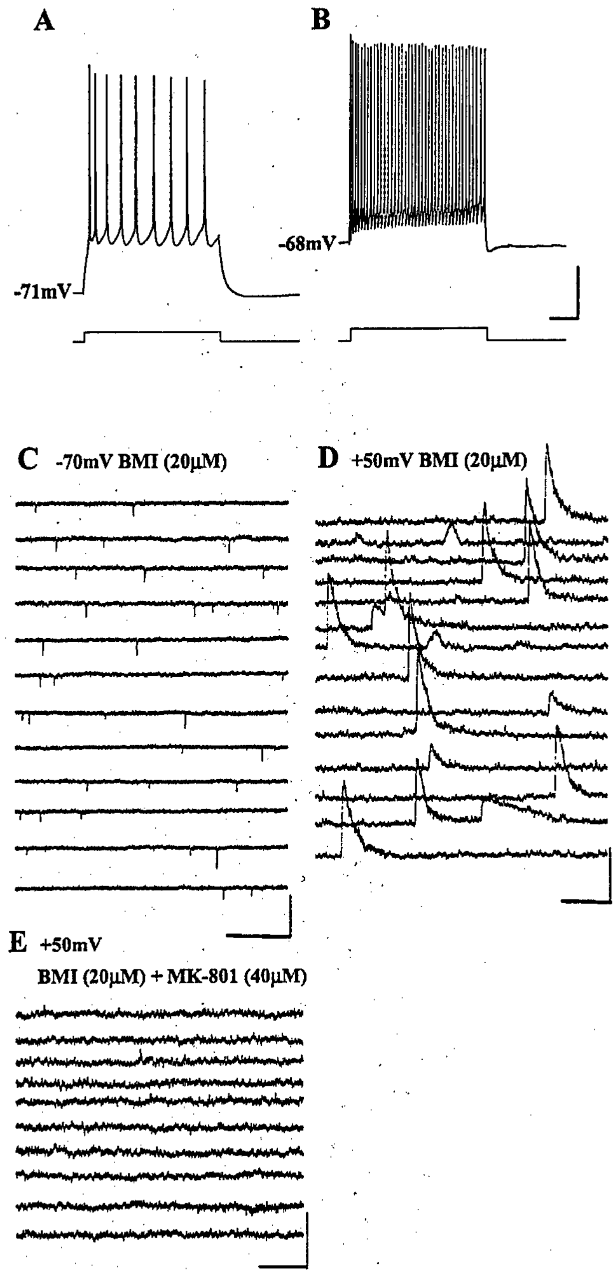

Figure 8. MK-801 blocks NMDA receptor-mediated EPSCs in PCC/RSC interneurons. *A*, Current-clamp recording of a PCC/RSC layer IV pyramidal cell in response to depolarizing current injection. The firing pattern of this pyramidal cell shows slow action potentials and frequency adaptation, characteristics typical for pyramidal cells. *B*, Current-clamp recording of a PCC/RSC layer III interneuron in response to depolarizing current injection. This interneuron fires rapidly and lacks frequency adaptation, characteristics typical for interneurons. Calibration: *A*, *B*, 200 msec, 20 mV, 80 pA. *C*, At the holding potential of −70 mV and in the presence of BMI (20 μM), DNQX-sensitive fast inward EPSCs are dominant in the interneuron. Calibration: 500 msec, 50 pA. *D*, When this interneuron was voltage-clamped at +50 mV and in the presence of BMI (20 μM), the long slow EPSCs were dominant. Calibration: 1000 msec, 100 pA. *E*, The slow EPSCs were blocked after administration of MK-801 (40 μM). Calibration: 1000 msec, 50 pA.

termine whether the decrease of sIPSCs and eIPSCs in pyramidal cells was caused by a reduction of action potential-dependent or action potential-independent mechanisms, we used TTX, a Na^+ channel blocker, to block synaptic events that are dependent on action potentials. Under such conditions, synaptic responses obtained in the presence of TTX are caused by quantal release and referred to as miniature IPSCS (mIPSCs).

We investigated the effect of MK-801 on mIPSCs in pyramidal cells in the PCC/RSC and found that MK-801 did not affect mIPSC frequency or amplitude. Figure 9*A* shows recordings of sIPSCs from a PCC/RSC pyramidal cell in layer III before and after application of TTX (0.5 μM). TTX caused a decrease in the mean sIPSC amplitude, indicating that a significant portion of the sIPSCs was caused by action potential-dependent GABA release from inhibitory interneurons. In the presence of TTX and at a holding potential of +30 mV, the average mIPSC frequency in the pyramidal cells was 4.5 ± 0.26/sec and the mean amplitude was 35 ± 6.2 pA (n = 5). After pretreatment of the slice with TTX for >30 min, bath application of MK-801 (40 μM) had no effect on the frequency (4.2 ± 0.23/sec) or amplitude (33 ± 5.8 pA) of mIPSCs (Fig. 9*A*). In contrast to the effects of MK-801 on spontaneous IPSCs, there were no significant changes in the cumulative probability distribution of either mIPSC frequency (Fig. 9*B*, *left panel*) or amplitude (Fig. 9*B*, *right panel*) after bath application of MK-801 (K-S; $p > 0.05$). These results indicated that MK-801 exerts its inhibitory effect on IPSCs of pyramidal cells through an action potential-dependent mechanism.

DISCUSSION

Using whole-cell patch-clamp techniques combined with morphological identification of neurons in rat brain slices, we have investigated the effect of the noncompetitive NMDA receptor antagonist MK-801 on sIPSCs and eIPSCs in pyramidal cells of PCC/RSC and parietal cortex. Our results demonstrate that in PCC/RSC pyramidal cells, $GABA_A$ receptor-mediated synaptic transmission is reduced by NMDA receptor antagonists. These observations suggest that NMDA receptor-mediated excitation of interneurons facilitates GABAergic inhibition of PCC/RSC pyramidal cells.

The modulation of GABAergic inhibition by MK-801 in two regions with different vulnerabilities to NMDA antagonist-induced neurotoxicity

MK-801 has neuroprotective effects in certain CNS disorders (Foster et al., 1988; Dirnagl et al., 1990). However, MK-801 also has negative effects *in vivo*, including altered behavior (Sams-Dodd, 1997) and temperature regulation (Colbourne et al., 1999). Several *in vivo* studies reported that chronic injection of low-dose MK-801 [or other NMDA receptor antagonists, e.g., phencyclidine (PCP)] causes neuronal vacuolization in the PCC/RSC (Olney et al., 1989; Fix et al., 1993; Corso et al., 1997). This vacuolization can be reversible at low doses; however, irreversible neuronal damage occurs after high doses or repeated low doses (Fix et al., 1993).

Given these results, a fundamental question arose: how could an agent that had been expected to be neuroprotective turn out to be neurotoxic? On the basis of their whole animal studies, Olney and others proposed that these drugs might impair neuronal inhibition, leading to hyperactivity and neuronal death (Olney et al., 1991; Corso et al., 1997; Horvath et al., 1997). However, neurotoxic mechanisms underlying this phenomenon may be complex and could include those mediated by direct disruption of

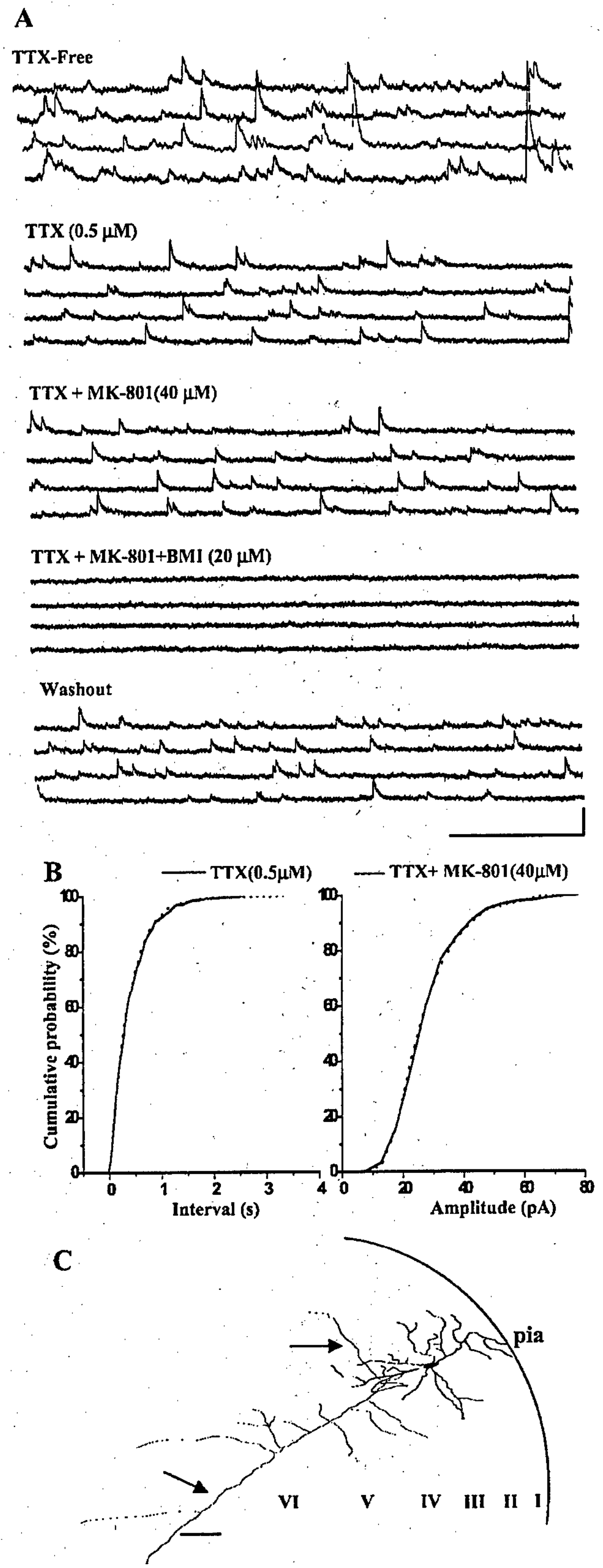

Figure 9. MK-801 does not suppress mIPSCs. *A*, In a PCC/RSC layer III pyramidal cell held at +30 mV, bath-applied TTX (0.5 μM) abolished all action potential-dependent IPSCs, leaving only mIPSCs. Bath-applied MK-801 (40 μM) had no effect on mIPSCs, whereas BMI (20 μM) reversibly blocked mIPSCs. Calibration: 500 msec, 0.05 nA. *B*, Cumulative mIPSC inter-event and amplitude distributions were not significantly changed (K-S test; $p > 0.05$), indicating that MK-801 produces disinhibition through an action potential-dependent mechanism. *C*, Camera lucida reconstruction of the same cell filled with biocytin. *I–VI*, Cortical lamina. *Arrows* denote axon. Scale bar, 100 μm.

GABAergic function (Olney et al., 1991) or through a more indirect mechanism via modulatory neurotransmitters acting in vulnerable regions [such as cholinergic (Olney et al., 1991;), serotoninergic (Loscher and Honack, 1991), adrenergic (Loscher and Honack, 1991; Farber et al., 1995a), and dopaminergic (Sharp et al., 1994; Farber et al., 1996). Either way, a significant loss of inhibition could result in hyperexcitability, and this could lead to excitotoxicity. Consistent with these proposals are studies in which MK-801 increased glucose utilization in regions vulnerable to neurotoxicity, including the cingulate and entorhinal cortices (Kurumaji and McCulloch, 1990; Nehls et al., 1990; Patel and McCulloch, 1995). These results suggest that the vulnerable areas were highly activated by MK-801. The use of brain slices allowed us to assess disinhibition with less interference from modulating inputs and to determine the extent of disinhibition produced within localized networks. In our study, we have found that NMDA antagonists greatly reduce IPSCs in the PCC/RSC, an area of high susceptibility to NMDA antagonist-induced neurotoxicity.

With respect to the regional vulnerability to NMDA antagonist-induced neurotoxicity *in vivo*, the PCC/RSC is more susceptible than other cortical areas detected with a range of methods, including hematoxylin and eosin, silver stains, and electron microscopy (Olney et al., 1989; Fix et al., 1993; Corso et al., 1997; Horvath et al., 1997). We have found regional differences in the disinhibitory effects of MK-801 *in vitro* that parallel *in vivo* histopathological studies. Specifically, PCC/RSC pyramidal sIPSCs were significantly decreased by MK-801, whereas parietal pyramidal sIPSCs were less affected. Similar to this weak effect of MK-801 on parietal sIPSCs, Salin and Prince (1996) demonstrated that D-APV, a competitive NMDA receptor antagonist, only minimally inhibited sIPSC frequency in rat somatosensory cortex, another area less vulnerable to MK-801-induced neurotoxicity (Olney et al., 1989, 1991). We therefore postulate that these regional differences in disinhibition may explain, at least in part, the regional difference in the vulnerability to NMDA antagonists-induced neurotoxicity.

Our data indicate that MK-801-induced reduction in excitatory synaptic input onto GABAergic interneurons could be responsible for reducing pyramidal cell sIPSC frequency, although this is indirect evidence. However, we also isolated NMDA receptor-mediated EPSCs in interneurons and found that MK-801 blocked these EPSCs, providing more direct evidence for NMDA antagonist-induced disinhibition. Entorhinal interneurons receive NMDA receptor-mediated input (Jones and Buhl, 1993); however, some hippocampal interneurons may not have NMDA receptors (McBain and Dingledine, 1993). Similar to the hippocampal interneurons (McBain and Dingledine, 1993; Mott et al., 1997), cortical interneurons have heterogeneous anatomic morphologies and physiological functions (Kawaguchi 1995; Kawaguchi and Kubota, 1996). These differences may contribute to regional vulnerabilities in MK-801-induced neurotoxicity.

Systemic administration of MK-801 results in altered field potentials, specifically limited to layer III of rat medial entorhinal cortex (Gloveli et al., 1997). A marked increase in immediate early gene c-*fos* expression in layer III was also noted in rats

pretreated with MK-801 (Vaisanen et al., 1999). These results suggested that cells in layer III are more sensitive to NMDA receptor antagonists. However, our experiments demonstrated that reduction in inhibitory transmission or disinhibition induced by MK-801 is not layer specific, although there are interlaminar differences in sIPSC frequency in rat somatosensory cortex (Salin and Prince, 1996).

MK-801 modulates pyramidal cell IPSCs via an action potential-dependent mechanism

Reduction of inhibitory transmission by NMDA antagonists has been observed in hippocampus (Hablitz and Langmoen, 1986; Grunze et al., 1996) and basolateral amygdala (Rainnie et al., 1991). In rat olfactory bulb, NMDA receptor activation is required for dendrodendritic inhibition (Schoppa et al., 1998). Direct infusion of D-APV bilaterally into the accessory olfactory bulb produced epileptiform seizures in adult female mice (Brennan and Keverne, 1989), an effect consistent with disinhibition. These investigators suggested that reduced inhibitory transmission could result from a loss of excitatory drive onto interneurons in the local circuit.

Our findings are compatible with this mechanism. MK-801 dramatically reduced the frequency of sIPSCs and the amplitude of eIPSCs but did not affect the frequency or amplitude of TTX-insensitive mIPSCs. This argues that MK-801 did not affect the postsynaptic efficacy of released GABA and did not affect quantal size or probability of spontaneous release. [Disinhibition produced by presynaptic mechanisms has been observed elsewhere, such as in studies of the effects of opioids and cannabinoids (Cohen et al., 1992; Hoffman and Lupica 2000)]. MK-801 seemed to affect action potential-dependent GABA release, compatible with an effect in inhibitory interneurons on either action potential-induced calcium influx into the presynaptic terminal or an effect on action potential initiation or propagation. Consequently, future experiments will focus on the effects of MK-801 on synaptic currents and resting spontaneous activity in interneurons.

Relation to proposed models

These results support the hypothesis that NMDA antagonists produce disinhibition within a PCC/RSC excitatory/inhibitory network. This disinhibition may be attributable partially to a local effect, because these findings were obtained in brain slices in which many of the modulatory input fibers have been severed.

These results, however, do not rule out the importance of inputs from other brain areas shown to play a role in NMDA antagonist-induced neurotoxicity [e.g., inputs from the anterior thalamus (Tomitaka et al., 2000) or cholinergic nuclei (Corso et al., 1997)]. This may be important, because it is not clear whether disinhibition alone is sufficient to produce neurodegeneration in the PCC/RSC. For example, if disinhibition is sufficient, then local injection of NMDA antagonists into the PCC or RSC might be expected to produce localized neurodegeneration. However, the results vary regarding the *in vivo* effects of direct injections of NMDA antagonists: D-APV injected directly into the cingulate caused neuronal vacuolization (Olney et al., 1989), whereas MK-801 injected into the RSC did not induce HSP70 heat shock protein, but instead, HSP70 was induced after bilateral (but not unilateral) MK-801 injections into the anterior thalamus (Tomitaka et al., 2000). Based on the latter findings, a model was constructed in which excitatory connections from the anterior thalamus to the RSC mediate MK-801-induced neurotoxicity, and it is within the thalamus that local modulation of GABAergic inhibition occurs (Tomitaka et al., 2000). That model is entirely consistent with their findings. However, our results suggest that there also is modulation of GABAergic function at the local cortical level. It is possible that locally mediated disinhibition (as seen in our slices) may not be sufficient to produce neurodegeneration. However, local disinhibition could become critical when modulated by other neurotransmitters or external excitatory inputs, then leading to full-blown neurodegeneration. Given that region-specific disinhibition seen *in vitro* does parallel region-specific neurodegeneration seen *in vivo*, it is plausible that disinhibition may be a candidate mechanism underlying neurotoxicity.

Clinical significance

These findings may have wide-ranging clinical significance because drugs with NMDA antagonist activity are being used clinically, and many more are under development. These include drugs for pain, epilepsy, and Parkinson's disease (Subramaniam et al., 1995; Boyce et al., 1999; Parsons et al., 1999). Also, certain abused drugs, such as PCP and ketamine, are NMDA antagonists (Olney et al., 1987). Potent NMDA antagonists such as PCP produce bizarre and disturbing behavioral effects in humans, but it has not been determined whether this reflects neurotoxicity. If so, it will be important to better understand the neurotoxic mechanisms and to reduce the risk of damage. One strategy involves using NMDAR-glycine site antagonists, which lack neurotoxicity (Berger et al., 1994; Auer, 1997). However, noncompetitive channel antagonists may still prove useful, so it will be important to better understand their neurotoxic mechanisms and the characteristics that increase vulnerability to neurotoxicity [e.g., age or gender (Auer, 1996; Wozniak et al., 1996)].

Also, NMDA antagonists are proposed to model psychiatric conditions such as schizophrenia (Ellison, 1995; Farber et al., 1995b). If there are shared mechanisms between drug-induced neurotoxicity and the etiologies of schizophrenia, these findings may have broad implications for psychiatry.

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Differential Effect of Ethanol on NMDA EPSCs in Pyramidal Cells in the Posterior Cingulate Cortex of Juvenile and Adult Rats

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Li, Qiang, Wilkie A. Wilson, and H. Scott Swartzwelder. Differential effect of ethanol on NMDA EPSCs in pyramidal cells in the posterior cingulate cortex of juvenile and adult rats. *J Neurophysiol* 87: 705–711, 2002; 10.1152/jn.00433.2001. Ethanol (EtOH) is a potent inhibitor of *N*-methyl-D-aspartate (NMDA) receptor–mediated activity in a number of brain areas, and recent studies have indicated that this inhibitory effect of ethanol is more powerful in the juvenile brain compared with the adult brain. However, previous direct developmental comparisons have been limited to studies of extracellular responses in the hippocampus. To begin an assessment of the mechanisms underlying this developmental sensitivity, we assessed the inhibitory effect of EtOH on NMDA receptor–mediated synaptic transmission in neocortical slices from adult (95–135 days old) and juvenile (28–32 days old) rats using the whole cell patch-clamp recording technique. In the presence of 6,7-dinitroquinoxaline-2,3-dione (20 μM) and bicuculline methiodine (20 μM), NMDA receptor–mediated excitatory postsynaptic currents were isolated from pyramidal cells of the posterior cingulate cortex (PCC). In slices from juvenile rats 5, 10, 30, and 60 mM EtOH reduced the mean amplitude of NMDA receptor–mediated EPSCs by 11, 22, 35, and 46%, respectively. However, the same concentrations of EtOH inhibited the mean amplitude of EPSCs by only 4, 8, 15, and 31% in slices from adult rats. This developmental difference in the potency of EtOH against NMDA receptor–mediated EPSCs was also observed when the holding potential of the neurons was increased to +30 mV, although the inhibitory effect of ethanol on adult neurons was diminished at that voltage. These results provide a cellular analysis of the enhanced potency of ethanol against NMDA receptor–mediated EPSCs in neocortical cells from juvenile animals compared with adults.

INTRODUCTION

The cognitive impairment that accompanies ethanol intoxication is a particular concern in young drinkers for several reasons. First, during the second decade of life people are generally engaged in educational activities that require the assimilation of large amounts of new information. Ethanol-induced compromises of learning and anterograde memory clearly compromise learning as suggested by the negative correlation between drinking and academic performance among students (Pullen 1994; Wechsler et al. 1995). In addition to memory impairment, the effects of ethanol on other higher-order cognitive functions such as judgment and response inhibition may predispose intoxicated individuals toward accidents and other negative sequelae of ethanol use such as fights, sexual assaults, and unprotected sex. Finally, the brain undergoes rapid development of neocortical synaptic circuitry throughout the second decade of life. This developmental change could alter neuronal responsiveness to acute doses of ethanol, and chronic exposure to ethanol during this period could potentially alter the developmental trajectory of neural circuits.

The effects of ethanol on neural signaling are varied and complex. However, it is now clear that *N*-methyl-D-aspartate (NMDA) receptor–mediated synaptic transmission is particularly sensitive to the acute (Lovinger et al. 1990; Morrisett and Swartzwelder 1993) and chronic (Snell et al. 1993) effects of ethanol. Given the linkage of NMDA receptor–mediated activity with learning and memory (Moser et al. 1998), impairment of this activity may be one mechanism whereby ethanol diminishes cognitive functions in vivo. The potency of ethanol against NMDA receptor–mediated neural activity, and its implications for the cognitive effects of ethanol, has sparked considerable interest. However, the cellular mechanisms of that potency remain obscure, and there is little information about ethanol potency at the cellular level outside the hippocampal formation.

It is now clear that the responsiveness of NMDA receptor–mediated neural activity to ethanol varies across development. The potency of ethanol as an antagonist of NMDA receptor–mediated population excitatory postsynaptic potentials (pEPSPs) (Swartzwelder et al. 1995a) and long-term potentiation (LTP) (Pyapali et al. 1999; Swartzwelder et al. 1995b) is greater in hippocampal slices taken from juvenile or adolescent rats compared with those taken from adults. In addition, hippocampally mediated spatial learning was impaired more potently by ethanol in adolescent rats compared with adults (Markweise et al. 1998). Interestingly, in humans an acute dose of ethanol has been shown to impair both verbal and figural learning more powerfully in people 21–24 yr of age than in those 25–29 yr of age (Acheson et al. 1998).

Although the developmental differences in ethanol potency are intriguing and may be of considerable value for health education, the cellular mechanisms underlying them remain virtually unexplored, and the extracellular effects have been assessed only in hippocampal tissue. The present study was

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designed to assess the potency of ethanol against NMDA receptor–mediated EPSCs in pyramidal neurons in the posterior cingulate cortex of juvenile and adult rats.

METHODS

Slice preparation

Neocortical slices were prepared from juvenile [*postnatal days 28–32* (*P28–32*)] and adult (*P95–135*) male Sprague-Dawley rats. The rats were anesthetized with isoflurane and decapitated. The brains of juvenile rats were quickly removed from the skulls and placed in cold (4°C) artificial cerebrospinal fluid (ACSF) containing (in mM) 120 NaCl, 3.3 KCl, 1.23 NaH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 1.8 $CaCl_2$, and 10 D-glucose at pH 7.3 (normal ACSF), previously saturated with 95% O_2-5% CO_2. The brains of adults were placed in a modified ACSF in which 1.8 mM $CaCl_2$ was replaced with 0.5 mM $CaCl_2$. Coronal cortical slices from both age groups containing posterior cingulate cortex (PCC) (Paxinos and Watson 1986) (300 μm thickness), were cut on a vibratome (Campden, model 752, England) and incubated in a holding chamber that contained normal ACSF that was continuously bubbled with 95% O_2-5% CO_2 at room temperature (22–24°C).

Whole cell voltage-clamp recording

Our whole cell patch-clamp techniques have been described in detail previously (Mott et al. 1999). For recording, patch pipettes were pulled from borosillicate glass capillary tubing (1.5 mm OD, 1.05 mm ID, World Precision Instruments, Sarasota, FL) on a Flaming-Brown horizontal microelectrode puller (model P-97, Sutter Instrument, Novato, CA). The pipettes were filled with an intracellular solution containing (in mM) 130 Cs-gluconate, 7 CsCl, 10 *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES), and 4 Mg-ATP (pH 7.25). The quaternary lidocaine derivative QX-314 (4 mM; Sigma, St. Louis, MO) was also included to suppress fast sodium currents. Osmolarity was adjusted to 280 mOsm. The pipette resistances generally were in the range of 4–7 MΩ. Biocytin (0.3 to ~0.4%; Sigma, St. Louis, MO) was also added to the intracellular solution for later visualization of the morphology of the recorded cells.

After 1 h of incubation in the holding chamber, a slice was transferred into a small submersion recording chamber at room temperature (22–24°C) and secured in place with a bent piece of platinum wire resting on the top of the slice. Individual cells were visualized using an infrared differential interference contrast (IR-DIC) Zeiss Axioskop microscopy and a ×40 water immersion objective. Tight seals (>1 GΩ) were obtained on pyramidal-shaped cells, and whole cell recordings were made after rupturing the cell membrane with gentle suction. The evoked NMDA receptor–mediated excitatory postsynaptic currents (EPSCs) were recorded continuously using an Axopatch 1D amplifier (Axon Instrument, Foster City, CA). Output current signals were DC-coupled to a digital oscilloscope (Nicolet model 410). Series resistance was monitored throughout the recording, and a cell was discarded if it changed significantly. The signal was further analyzed using Strathclyde Electrophysiology Software, Whole Cell Program (Courtesy of Dr. John Dempster) with an interface (BNC-2090, National Instruments, Austin, TX) to a PC-based computer.

Electrical stimulation and isolation of NMDA receptor–mediated EPSCs

A monopolar tungsten electrode (A-M System, Carlsborg, WA) was placed about 50 to ~70 μm from the soma of the recorded pyramidal cells. In the presence of $GABA_A$ receptor antagonist bicuculline methiodine (BMI; 20 μM) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μM), NMDA receptor–mediated EPSCs were evoked by electrical stimulation. The stimulus threshold was first determined by increasing intensity of constant current rectangular wave pulses generated by an isolated stimulator (Grass S88, Grass Instrument, Quincy, MA) until detectable responses occurred. Then constant current rectangular stimulus pulses 50% higher than threshold intensity with duration of 0.1 ms and interval of 0.033 Hz were delivered through the electrode. After the baseline measurements were established in control ACSF, bath application of 5, 10, 30, and 60 mM of ethanol (EtOH) was initiated. These concentrations of ethanol were chosen because they correspond with human blood ethanol concentrations across a range of very mild to very heavy intoxication. The evoked NMDA receptor–mediated EPSCs were continuously monitored for 15 min followed by a 30-min wash out period.

Histological identification of PCC pyramidal neurons

During recording, neurons were filled with biocytin. After the end of the recording, the slices remained in the recording chamber for an additional 10–20 min to allow biocytin transport within the axon. The slices were then placed overnight in 4% paraformaldehyde, 0.05% gultaraldehyde in 0.1 M phosphate buffer saline (PBS). They were then washed thoroughly in PBS and incubated in 0.1 M Tris-buffered saline (TBS) containing 1% H_2O_2 for 30 min. The slices were then incubated with avidin-biotin-peroxidase complex (ABC kit, Vector Labs, Burlingame, CA) in TBS containing 0.05% Triton X-100 overnight at 4°C, rinsed three times in PBS, and then reacted in a solution containing DAB (DAB kit, Vector Labs, Burlingame, CA).

The slices were then cleared and mounted. The morphology of the biocytin-filled pyramidal cells was examined under light microscope, and neurons were drawn using a camera lucida.

Statistical analysis of data and drug applications

The data were analyzed off-line using the Strathclyde Electrophysiological Software. Two-way analyses of variance (ANOVA) were used to assess age and dose effects, and Student's *t*-tests were used post hoc where appropriate. The significance level was set to $P < 0.05$ for all statistical tests. All grouped data are presented as means ± SE in the figures. All drugs were applied via bath superfusion. DNQX and BMI were purchased from Sigma (St. Louis, MO). D-(−)-2-Amino-5-phosphonovaleric acid (APV) was from ACROS (Geel, Belgium).

RESULTS

NMDA receptor–mediated EPSCs

Whole cell recordings were obtained from 45 morphologically identified pyramidal cells in the PCC. The recorded PCC pyramidal cells were located in the layers II–V. Among these cells, 25 neurons were from slices from juvenile rats, and 20 were from slices from adults. The resting membrane potentials (determined immediately after rupturing the cell membrane) of PCC pyramidal cells recorded from juvenile (−68.7 ± 0.63 mV, mean ± SE, $n = 13$) and adult (−67.9 ± 0.55 mV, $n = 12$) rats were not significantly different. In addition, current-voltage (*I-V*) curves constructed from PCC pyramidal cells in the presence of 1.2 mM Mg^{2+} indicated that NMDA receptor–mediated EPSCs reversed at 4.4 ± 0.6 mV in juvenile rats ($n = 10$) and at 5.1 ± 0.57 mV in adult rats ($n = 8$), similar to those observed in the somatosensory cortex of rats (Kim et al. 1995) and the hippocampus of mice (Kirson and Yaari 1996). There was no significant difference between these reversal potentials,

indicating that there is no developmental change in EPSC reversal potential in slices from juvenile compared with adult rats.

Figure 1 shows evoked NMDA receptor–mediated EPSCs recorded from two PCC pyramidal cells from juvenile (28–32 days old) rats. In the presence of DNQX and BMI, electrical stimuli evoked a slow inward current in pyramidal cells that were held at −30 mV (Fig. 1*A, left panel*). The evoked currents in pyramidal cells were completely abolished by bath application of 50 μM APV in all cells recorded from juvenile rats, indicating these currents were mediated by NMDA receptors. Furthermore, the evoked NMDA EPSCs became outward

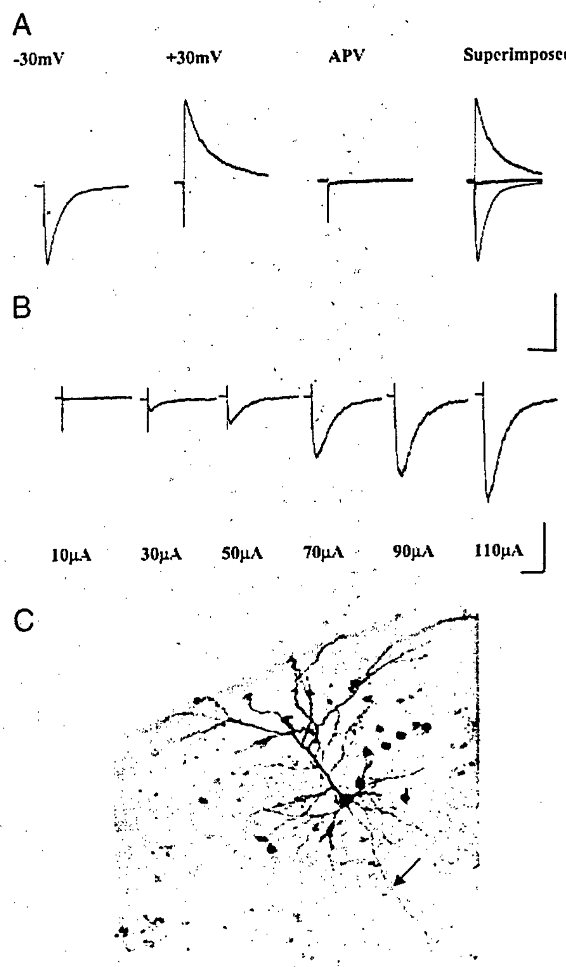

FIG. 1. *N*-methyl-D-aspartate (NMDA) receptor—mediated excitatory postsynaptic currents (EPSCs) isolated from posterior cingulate cortex (PCC) pyramidal cells in slices from juvenile rats. *A*: in the presence of 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μM) and bicuculline methiodine (BMI; 40 μM), a single pulse evoked an inward current from a PCC pyramidal cell from a 29-day-old rat at a holding potential of −30 mV. The current became outward when the holding potential was raised to +30 mV. The slow current was completely blocked by bath application of 50 μM D-(−)-2-amino-5-phosphonovaleric acid (APV). Calibration bars, 200 mS and 100 pA. *B*: in another PCC cell, gradual increases of electrical stimulus intensity result in a graded increase in the amplitude of NMDA receptor–mediated EPSCs. Evoked EPSCs were recorded when cell was held at −30 mV. Calibration bars, 200 ms and 100 pA. *C*: microphotograph showing the morphology of the cell whose electrophysiology was shown in *A*. Axon is indicated by an arrow. Scale bar, 30 μm.

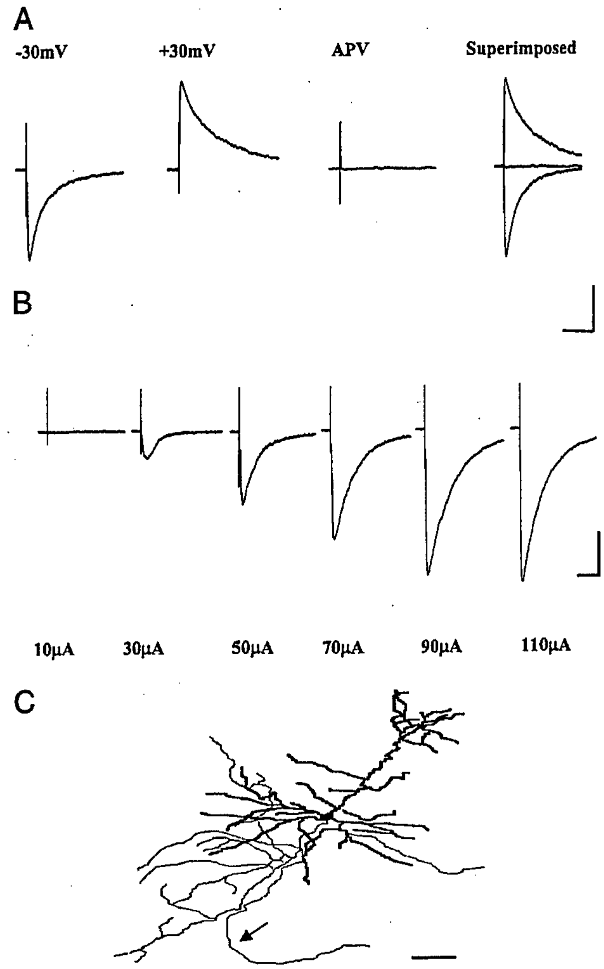

FIG. 2. NMDA receptor–mediated EPSCs isolated from PCC pyramidal cells in slices from adult rats. *A*: in the presence of DNQX (20 μM) and BMI (20 μM), a single pulse evoked an inward current from a PCC pyramidal cell from a 129-day-old rat at a holding potential of −30 mV. The current became outward when the holding potential was raised to +30 mV. The slow current was completely blocked by bath application of 50 μM APV. Calibration bars, 200 mS and 100 pA. *B*: in another PCC cell, gradual increases of electrical stimulus intensity result in a graded increase in the amplitude of NMDA receptor–mediated EPSCs. Evoked EPSCs were recorded when cell was held at −30 mV. Calibration bars, 200 ms and 100 pA. *C*: camera lucida reconstruction of biocytin-filled neuron whose electrophysiology was shown in *A*. Axon is indicated by an arrow. Scale bar, 30 μM.

when the cell was held at +30 mV. At the holding potential of −30 mV, increases of the stimulus intensity from 10 to 110 μA evoked EPSCs of increasing amplitude in another pyramidal cell (Fig. 1*B*). Figure 1*C* shows the morphology of the biocytin-filled pyramidal cell that generated the EPSCs shown in Fig. 1*A*.

Figure 2 shows NMDA receptor–mediated EPSCs recorded from two PCC pyramidal cells in slices from adult (95–135 days old) rats. Similar to those recorded from the juvenile rats, the evoked NMDA receptor–mediated EPSCs were completely blocked by D-APV (50 μM), and electrical stimuli elicited an inward and outward current when the cell was held at −30 and +30 mV, respectively (Fig. 2*A*). A similar input-output curve was observed for the EPSCs across the range of increasing electrical stimulus intensities described above (Fig. 2*B*). Figure

2*C* shows a camera lucida reconstruction of the pyramidal cell whose physiological responses were shown in Fig. 2*A*.

Effects of EtOH on NMDA receptor–mediated EPSCs

Ethanol inhibited NMDA receptor–mediated EPSCs in a concentration-dependent manner ($F_{[3]} = 144.34$, $P < 0.0009$), and did so more potently in pyramidal cells from juvenile rats than in those from adults ($F_{[1]} = 14.47$, $P = 0.002$). There was also a significant age by concentration interaction ($F_{[3]} = 4.26$, $P = 0.01$), indicating that the concentration-response effects varied with the age of the animals from which slices were taken. Post hoc comparisons indicated that the inhibitory potency of ethanol was significantly greater in PCC neurons from juveniles compared with adults at each of the EtOH concentrations tested. However, there was no significant difference between the amplitude of EPSCs across age groups either at baseline or after wash out of ethanol.

In the presence of DNQX, BMI and 1.2 mM Mg^{2+}, the effects of EtOH on NMDA receptor–mediated EPSCs were assessed when the cells were held at −30 mV. Local electrical stimulation evoked an inward EPSC in PCC pyramidal cells in both age groups. Figure 3 shows the inhibitory effects of a range of EtOH concentrations on NMDA receptor–mediated EPSCs recorded from a PCC pyramidal cell from a juvenile rat (*top panel*) and an adult rat (*bottom panel*). In these experiments, after a 10-min baseline recording period, EtOH concentrations of 5–60 mM were sequentially bath-applied, and the effects of each concentration of EtOH was observed for 15 min. In cells from juvenile rats, the EPSC amplitude began to decrease after application of 5 mM EtOH, and the decreases were concentration-dependent across the range of concentrations applied. At concentrations of 5, 10, 30, and 60 mM EtOH, this cell's peak EPSC amplitude decreased by 11, 20, 35, and 47%, respectively, relative to baseline. The inhibitory effect of EtOH on NMDA receptor–mediated EPSCs was reversible after a 30-min wash out with normal ACSF. The EPSCs recorded from the adult neuron shown in Fig. 3 (*bottom panel*) were markedly less inhibited by ethanol across the range of concentrations tested. In this neuron concentrations of 5, 10, 30, and 60 mM EtOH decreased the peak amplitude of the NMDA EPSC by 4, 9, 24, and 36%, respectively relative to baseline. Again, the inhibitory effect was reversed after wash out. In addition, the pyramidal cell from the juvenile rat in the *top panel* of Fig. 3 shows a graded reduction in the amplitude with successive application of each concentration of EtOH. In contrast, the EPSC from the pyramidal cell of an adult rat (Fig. 3, *bottom panel*) showed a marked decrease in peak amplitude only after bath application of 30 mM EtOH. A further reduction was observed after 60 mM EtOH was bath applied.

Ethanol (5–60 mM) reduced NMDA receptor–mediated EPSC amplitudes in an age- and concentration-dependent manner. Figure 4 shows the averaged inhibitory effects of different concentrations of EtOH on NMDA receptor–mediated EPSCs from juvenile and adult neurons. In slices from juvenile rats, 5, 10, 30, and 60 mM EtOH reduced the mean amplitude of NMDA receptor–mediated EPSCs by 12 ± 2.0% ($n = 12$), 23 ± 3.5% ($n = 12$), 36 ± 3.7% ($n = 11$), and 46 ± 2.7% ($n = 13$), respectively, relative to baseline. However, the same concentrations of EtOH inhibited the responses by only 4 ± 0.9% ($n = 8$), 8 ± 1.2% ($n = 7$), 15 ± 3.1% ($n = 8$), and 31 ± 4.1% ($n = 8$) in slices from adult rats relative to baseline ($P < 0.05$). When the depressant effects caused by the same concentration of EtOH was compared between the two age groups, there are significant differences in the amplitude reduction of NMDA receptor–mediated EPSCs at each concentration tested, but not at baseline or after the wash out of ethanol.

To determine whether the effects of higher ethanol concentrations were related to either a cumulative inhibitory effect, or to acute tolerance, we compared the effects of a single application of 60 mM EtOH with the effects of 60 mM EtOH when presented at the end of a series of ethanol concentrations as described above. There were no significant differences between the effects of 60 mM EtOH presented in these ways. In juvenile rats, a single dose of 60 mM EtOH decreased the amplitude of NMDA EPSCs by 46.2 ± 2.7% ($n = 12$) compared with a reduction of 45.9 ± 1.9% ($n = 7$) observed after sequential application of 5, 10, and 30 mM EtOH prior to 60 mM EtOH. There was no

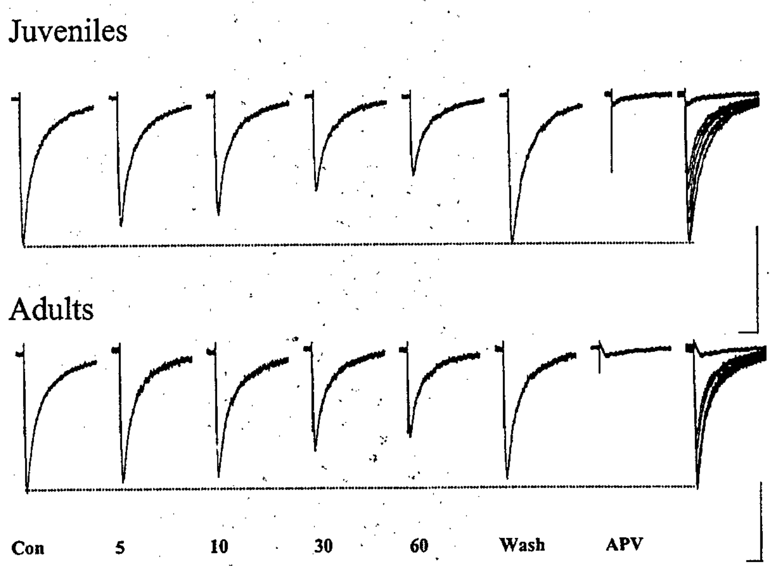

FIG. 3. Inhibitory effect of a range of EtOH concentrations on NMDA receptor–mediated EPSCs in PCC neurons from juvenile (*top panel*) and adult (*bottom panel*) rats. The inhibitory effects were reversed after wash out of EtOH from the artificial cerebrospinal fluid (ACSF). The holding potential was −30 mV for all recordings. Calibration bars, 200 ms and 100 pA.

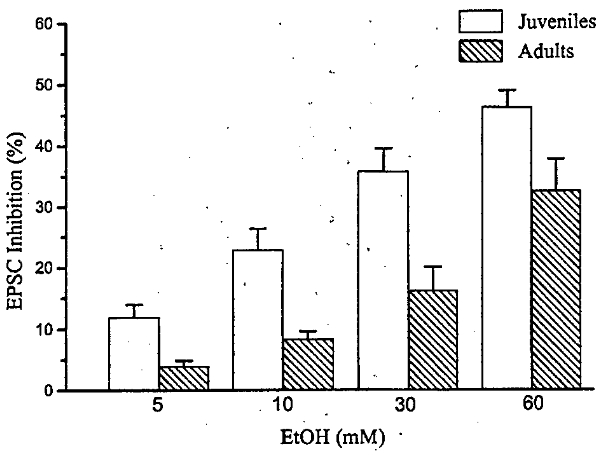

FIG. 4. Differential inhibitory effects of a range of EtOH concentrations on NMDA receptor–mediated EPSCs in PCC neurons from juvenile and adult rats. Bars represent the mean (±SE) percent of inhibition relative to baseline EPSC amplitude at 5–60 mM EtOH in neurons from juvenile (open bars) and adult (filled bars) rats. All recordings were made at −30 mV.

significant difference in the averaged amplitude of NMDA EPSCs under these conditions. Similarly, in slices from adult rats, a single dose of 60 mM EtOH reduced the amplitude of NMDA EPSCs by 32.6 ± 5.1% (n = 9) compared with 32.7 ± 3.7% (n = 6) from the cells exposed to sequential applications of 5, 10, and 30 mM EtOH prior to 60 mM EtOH. There was also no significant difference in the averaged amplitude of NMDA EPSCs under these conditions in the slices from adult rats (unpaired t-test, $P >$ 0.05).

Previous studies of extracellular recordings have indicated that the antagonism of NMDA receptor–mediated potentials in hippocampal slices by ethanol is increased in the presence of Mg^{2+} (Morrisett et al. 1991). Thus it is possible that differential regulation of the NMDA channel by Mg^{2+} could account for the different potency of ethanol against NMDA receptor–mediated EPSCs. As an initial assessment of this possibility, we measured the effects of EtOH on NMDA receptor–mediated EPSCs while holding the neurons at +30 mV to remove the blockade of the NMDA receptor channel by Mg^{2+}. As shown in Fig. 5, 60 mM EtOH still caused a greater decrease in EPSC amplitude in pyramidal cells from juvenile rats (Fig. 5, *A* and *C*) compared with those from adults (Fig. 5, *B* and *C*). At the holding potential of +30 mV, 60 mM EtOH reduced the amplitude of NMDA receptor–mediated EPSCs by 43.7 ± 5.4% (n = 6) relative to baseline in neurons from juvenile rats (Fig. 5*C*), whereas the same concentration of EtOH only inhibited the amplitude of NMDA EPSCs by 21.6 ± 4.6% (n = 5) relative to baseline in cells from adult rats (Fig. 5*C*). This difference was statistically significant ($t_{[9]} = 3.00$, $P = 0.01$). As expected, relief of the Mg^{2+} block of the NMDA channel diminished the inhibitory effect of ethanol in neurons from adult animals. At −30 mV the percent inhibition produced by 60 mM EtOH was 32.6%, whereas at +30 mV the percent inhibition was 21.6% ($t_{[12]} = 2.47$, $P = 0.03$). However, there was no change in the inhibitory effect of ethanol against NMDA receptor–mediated currents in neurons from juveniles at +30 mV.

DISCUSSION

There are several findings from this study. First, NMDA receptor–mediated EPSCs were more sensitive to the inhibitory effect of ethanol in neocortical neurons from juvenile rats than in those from adults. Although we observed concentration-dependent inhibitory effects of ethanol on EPSCs in cells from both age groups, the inhibitory potency of ethanol was significantly greater among cells from juvenile animals. In addition, the difference in inhibitory potency did not appear to be mediated by either sensitization or acute tolerance, and increasing the holding potential to +30 mV did not alter the difference in inhibitory potency of a high concentration of ethanol, suggesting that it did not depend on an interaction with Mg^{2+} within the NMDA receptor channel. Finally, from a purely developmental perspective, we found that there was no difference in the reversal potential of NMDA receptor–mediated EPSCs in cells from juvenile animals compared with those from adults.

To our knowledge this is the first demonstration of an age-dependent difference in ethanol potency against NMDA

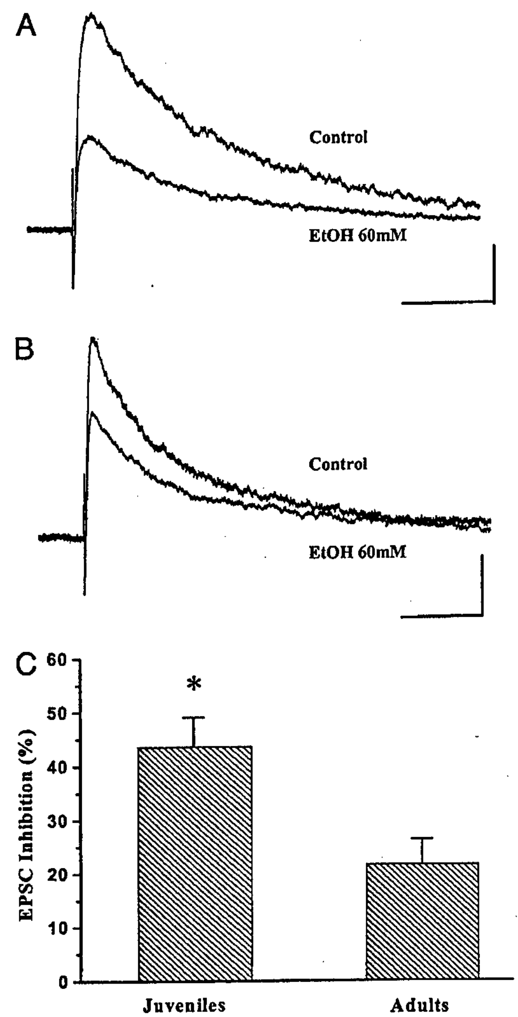

FIG. 5. Inhibitory effect of 60 mM EtOH on NMDA receptor–mediated EPSCs in neocortical cells from juvenile (*A* and *left bar* of *C*) and adult (*B* and *right bar* of *C*) rats at a holding potential of +30 mV. Calibration bars in *A* and *B*, 200 ms and 100 pA. * The mean percentage of inhibition was significantly different between the 2 groups of cells (unpaired t-test, $P < 0.05$).

receptor–mediated EPSCs in neocortical neurons. Previous studies using extracellular recordings have shown that ethanol more potently inhibits NMDA receptor–mediated pEPSPs (Swartzwelder et al. 1995a) and the induction of LTP (Pyapali et al. 1999; Swartzwelder et al. 1995b) in hippocampal area CA1 in brain slices from juvenile animals than in those from adults. Because of the well-known effects of acute ethanol exposure on learning, the hippocampal formation has been a region of consistent study within the ethanol literature. The inhibitory effect of ethanol against NMDA-mediated activity may be related to the learning impairments observed after acute ethanol exposure. That the effect is greater in hippocampi from juvenile and adolescent animals is consistent with the observed developmental differences in learning impairment during ethanol exposure in both humans (Acheson et al. 1998) and animal models (Markweise et al. 1998).

The present results indicate that enhanced sensitivity to ethanol in juveniles may extend to cognitive domains beyond learning and memory. The posterior cingulate cortex has been implicated in a number of neuropsychiatric disorders and is thought to mediate some subtle behaviors in humans related to spatial orientation, memory, and monitoring shifting cognitive orientation (Vogt et al. 1992). It also appears to be sensitive to the effects of chronic ethanol exposure. Such exposure in mice decreased the number and changed the morphology of PCC neurons (Marrero-Gordillo et al. 1998). As in other cortical areas, most excitatory synapses in the cingulate are thought to be glutamatergic, and include both AMPA and NMDA receptor subtypes (Hestrin 1996). The NMDA receptor/channel complex allows calcium entry; this initiates a cascade of cellular signals, some of which mediate synaptic plasticity, but also may mediate neurotoxicity (Rothman and Olney 1995). Thus the enhanced sensitivity of the pyramidal cells in this region to ethanol during juvenile development could indicate heightened vulnerability of these neurons to ethanol-mediated excitotoxicity. This could have broad implications for cognitive development in young individuals who drink ethanol in substantial amounts.

In addition to its focus on neocortical neurons, another unique feature of the present study is the cellular level of analysis. Previous direct studies of developmental sensitivity to ethanol used extracellular recordings from hippocampal slices (Pyapali et al. 1999; Swartzwelder et al. 1995a,b). Although these were valuable demonstrations of the developmental sensitivity to ethanol, it remained unclear whether the differences were due to sensitivity at the neuronal level or whether circuit interactions were the salient point difference. The present results clearly indicate that neocortical neuronal responsiveness to ethanol varies across the juvenile to adult period in rats, and these results are consistent with those from the hippocampal formation. The cellular level of analysis also provided the opportunity to compare the input resistance of NMDA EPSCs in slices from juvenile and adult animals. The fact that there was no significant difference rules out that aspect of membrane function as a mechanism underlying the increased sensitivity of juvenile neurons to ethanol.

The whole cell technique also afforded us the opportunity to test the inhibitory effects of ethanol on NMDA receptor–mediated EPSCs at multiple holding potentials. While the developmental difference in the inhibitory potency of a single high concentration of EtOH (60 mM) remained intact when the holding potential was increased from −30 to +30 mV, the voltage change diminished EtOH-induced inhibition in neurons from adult animals while those from juveniles were not affected. The decrease of EtOH-induced inhibition in adult tissue is consistent with previous reports using hippocampal slices in which reductions of Mg^{2+} diminished the inhibitory effect of EtOH on NMDA receptor–mediated pEPSPs (Morrisett et al. 1991). However, a direct comparison of this effect between juvenile and adult neurons has never been made. The lack of such an effect on neurons from juvenile animals could indicate that EtOH-induced inhibition of NMDA currents is not regulated by Mg^{2+} in juvenile neurons. Among hippocampal pyramidal cells, Mg^{2+} is a less potent regulator of NMDA receptor–mediated EPSPs in juvenile rats compared to adults (Morrisett et al. 1990). Thus Mg^{2+} regulation of the channel is different in juveniles compared with adults and could be of mechanistic significance for the developmental sensitivity of NMDA receptor–mediated activity to ethanol.

The present findings add to a rapidly expending literature on the unique potencies of ethanol against various neurophysiological and behavioral outcomes during postnatal development. We have shown that the enhanced sensitivity of NMDA receptor–mediated electrophysiological activity in the developing CNS extends to the neocortex and is observable at the cellular level. These results may figure productively in a very important and current dialogue in the U.S. related to alcohol consumption by young people. The use of ethanol by juveniles and adolescents is a consistent and growing problem. As we learn more about the parameters and mechanisms underlying the differences in developmental sensitivity to EtOH, this line of research may inform the public discussion on alcohol education and policy.

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